THE OXIDATION OF CYSTEINE AND CYSTINE

By

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THE OXIDATION OF CYSTEINE AND CYSTINE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

This thesis consists of three studies of the oxidation behavior of cysteine and cystine, which are compounds of considerable biological importance. The first of these studies deals with the oxidation of cysteine to cystine with ferricyanide. It had been observed that this reaction was inhibited by the addition of ethylenedinitrilotetraacetic acid (EDTA), a good complexing agent (14). A further study of this phenomenon constitutes the first part of the thesis; the study includes the effect of EDTA on two other mercaptans. A paper that describes the principal results of this work has been published and is reproduced in Appendix A. Chapter III presents some additional details and other material that seems worth reporting but that was not included in the paper. Although this work gave some interesting results, it was decided that further work on the kinetics of the cysteine-ferricyanide reaction would not be rewarding.

The investigation of another problem was therefore undertaken, that of oxidizing cysteine and cystine to cysteic acid. This reaction is of great utility in the determination of protein structure. Two reagents were chosen for study: iodate and periodate. They have a strong oxidizing action and they are easily determined analytically. The second portion of this thesis reports the reaction of iodate with cysteine and cystine. The rates and stoichiometry of the reaction were

studied under several conditions; in some conditions, rapid and quantitative conversion to cysteic acid could be realized. The principle findings are given in a form suitable for publication in the Journal of the American Chemical Society; this constitutes Appendix B. Chapter IV of the thesis gives additional details.

It was then decided to investigate the reaction of iodate with a protein, insulin, which contains three disulfide bonds. This work is described in Chapter V.

The third portion of the thesis deals with the reaction of periodate: the rates and stoichiometry were studied under various conditions. The results first obtained indicated that the application of this reagent to proteins was more complex than that of iodate. For this reason, this investigation was not developed fully. The results are described in Chapter VI.

Chapter II presents reviews of the pertinent literature.

CHAPTER II

REVIEW OF THE LITERATURE

This review is divided into sections, each of which has reference to the subjects treated in the chapters that follow. In order to make the connection clear, the sections below are entitled in the same manner as the chapter to which they principally refer. The only exception is the second section which discusses the oxidation of cysteine and cystine to higher products by reagents not involving iodine.

Inhibition and Catalysis in the Oxidation of Cysteine by Ferricyanide

The oxidation of cysteine, which is a mercaptan, may produce the disulfide, cystine, or higher oxidation products. Some oxidizing agents may give a mixture of both. The discussion in this section will be primarily devoted to the former type of reaction except those in which the oxidant involves iodine. The formation of higher oxidation products will be discussed in the next section. Iodine and iodate which may give either type of product, are discussed separately in the third section.

The oxidations of cysteine and other mercaptans by a variety of reagents has been reviewed by Pascal (90). Some of these are porphyrindin (11,48), tetrathionate (5,45), O-iodosobenzoic acid (52), and alloxan (116). These reagents have found some application in the study of mercapto groups in proteins. These reactions are discussed in the

reviews by Barron (12), Cecil (20), Cecil and McPhee (22), Tarbell (115), Martin and Synge (81), and Chinard and Hellerman (27). The use of ferricyanide is well discussed in a review by Herriot (53). Another substance which readily oxidizes cysteine to cystine is molecular oxygen (83,130).

The use of ferricyanide as an oxidizing agent for sulfhydryl groups in proteins was introduced by Anson (4). He made several studies on its reactions with the mercapto groups of egg albumin (4,5) and tobacco mosaic virus (7). In one article (6) he indicates that the reaction is catalyzed by added copper ion and inhibited by the addition of cyanide. In another article, coauthored by Mirsky (86), he indicates that certain other protein groups may also be oxidized by ferricyanide. The reaction of ferricyanide with ovalbumin has been studied by Katyal et al. (60). The inhibitory effect of Versene mentioned in this article formed part of the basis for the present work. Lontie and Beckers (73) reported no reaction with the native protein, in accordance with other workers, and found 2 to 3 groups after denaturation. They also describe the reaction to ferricyanide with other proteins; lysozyme (73), three serum albumins (74), and amandin (72). They find that amandin requires more of ferricyanide than of several other mercapto group reagents and again discuss the possibility of reaction with groups other than the mercapto; they mention tryptophan and tyrosine as possibilities.

Another literature reference of interest is the determination of cysteine by amperometric titration (129); it was found that several common amino acids, including tryptophan, do not interfere. Mason (82) used ferricyanide to determine glutathione; this method can be applied in tissues. Baker et al. (10) have used ferricyanide to determine the

sulfhydryl content of doughs.

The catalytic effect of metal ions mentioned by Anson (6) and Katyal et al. (60) has not been extensively studied. A recent paper (126) has appeared which discusses the inhibitory effect of ethylenediaminetetraacetic acid (EDTA) on the oxidations of cysteine and glutathione. These authors also studied several other oxidizing agents and compare the effect for one and two electron acceptors. However, their conclusions are somewhat different from those which are presented in Appendix A (46).

The reactions of ferricyanide with numerous other organic mercapto compounds have been studied. Some of these include thiophenol (61), 3-mercaptopropionic acid (16), and 1-octanethiol (62). The latter two are studied in this work. For 3-mercaptopropionic acid, the possibility of catalysis or inhibition is not mentioned. For 1-octanethiol, the inhibitory effect of cyanide is discussed but is explained by a mechanism other than catalysis. These questions are examined in Chapter III.

The Oxidation of Cysteine and Cystine to Higher Products

As stated in the preceding section, cysteine may be oxidized to higher products than cystine and cystine itself may also be oxidized. The use of iodine-containing reagents for this oxidation is left for the next section. This section will deal with the use of non iodine-containing reagents for the production of such higher products.

Many of the reviews mentioned in connection with the oxidation of mercaptans to disulfides also discuss further oxidations (12,22,27,53, 81). The possible higher oxidation states are summarized in Table II-1.

TABLE II-1

OXIDATION STATES DERIVED FROM CYSTEINE AND CYSTINE

CySOH	Cysteinesulfenic Acid
$CySO_2H$	Cysteinesulfinic Acid
$CySO_3H$	Cysteic Acid
CySOSCY	Cysteine Sulfoxide
CySOSOCy $CySO_2SCy$	Cystine Disulfoxide
$CySO_2SOCy$	Cystine Sulfoxide Sulfone
$CySO_2SO_2Cy$	Cystine Disulfone

The production of cysteic acid is a primary objective of the present thesis and will accordingly receive the most attention. However, the other products will be mentioned also.

The conversion of cysteine and cystine to the higher oxidation products requires a stronger oxidizing agent than the oxidation of cysteine to cystine. The reagent most widely used for the conversion to cysteic acid is performic acid, introduced by Toennies and Homiller (117). This reagent is also capable of oxidizing disulfide and mercapto groups in proteins to sulfonic acid groups. Insulin is a good example (99). Schram et al. (103) utilize this oxidation for the determination of cystine in proteins; after acid hydrolysis, which cysteic acid can undergo with only a little decomposition, the cysteic acid is separated by chromatography and is estimated.

A very closely related oxidant which will convert cysteine and cystine to cysteic acid is peracetic acid (131). Some of the intermediates in this reaction, produced by incomplete oxidation, have been

studied (76). The oxidation of keratin by peracetic acid has been described (2).

Hydrogen peroxide can give oxidation products higher than cystine (30), especially if a catalyst is used, such as a vanadic acid sol (43). Another reagent is bromine in water (30,93). Cystine may also be oxidized to cysteic acid by perchlorate under certain conditions (118,119). Under somewhat different conditions, cystine in perchloric acid may be oxidized to cystine disulfoxide with perbenzoic acid (121). Some of the properties (120) and reactions (64), especially dismutation of the cystine disulfoxide have also been studied. Another reagent is thallic sulfate which oxidizes cystine to cysteic acid in a second order reaction (91). Finally, according to Andrews (3), air may oxidize cystine to cysteic acid plus some sulfate; the reaction occurred in HCl but not in H_2SO_4 solutions. Shinohara and Kilpatrick (110) reported finding increasing amounts of cysteine and cysteic acid in acid solutions of cystine which had been standing for 6 hours or more.

Reaction of Cysteine and Cystine with Iodate

As stated earlier, the reactions of both iodate and iodine will be discussed in this section. A third iodine containing species, periodate, will be discussed in the final section.

The use of iodine for the determination of cysteine by oxidation was introduced by Okuda (93). This method involves the reaction with KIO_3 in a medium which contains excess iodide and acid, which releases iodine. Okuda (94) later extended the method to the determination of cystine by first reducing it with zinc dust in HCl and then by the iodometric determination of the cysteine produced. Many applications have

been made of this method, which will not be reviewed in detail. Some review of these applications appears in the reviews mentioned in the first section. Only a few pertinent works will be discussed here.

Dowler (36) states that iodine solutions do not oxidize cysteine to cystine at low concentrations, but does not specify the product. Later Yamazki (135) states that potentiometric titration of cysteine with $NaIO_3$ in the presence of iodide is more accurate than the titration with bromate since the formation of cystine is favored over "cysteinic acid" (probably cysteic acid); he mentions that tryptophan also is oxidized and that temperature and acidity are very important. Lucas and King (75) used direct titration with iodine for the determination of cysteine and found it better than Okuda's procedure. Shinohara (107) studied Okuda's procedure in detail and found that excess iodine is consumed; he then studied the reaction of iodine with cystine and showed that cystine is very slowly oxidized to cysteic acid in acid solution. He later made a kinetic study of the reaction (108) and discusses a possible mechanism involving the stepwise oxidation to CySOH, $CySO_2H$, and finally $CySO_3H$ (109). Simonsen (111) reported that cysteine sulfinic acid was formed and could be isolated when a cysteine solution was added slowly to an iodine solution.

It is the author's opinion that in view of the report that cysteine is formed upon the standing of an acidic cystine solution, and Simonsen's oxidation of cysteine to higher products when the cysteine is added to the iodine which is equivalent concentrationwise to the production in solution of cysteine, the oxidation of cystine to iodine may well be an apparent one actually being the oxidation of cysteine.

Lavine (63) shows that the oxidation of cysteine by iodine could

be made to give only cystine by conducting it in iM KI and 1M HCl. He mentioned earlier work which showed that cystine disulfoxide (67) and cysteinesulfinic acid (104) are reduced to cystine in this medium. Baernstein (9) used hydriodic acid to hydrolyze proteins; cystine was thus reduced to cysteine, which could then be simply determined by Okuda's method. Other pertinent articles include one by Chen (26) who compares Okuda's direct (KIO_3 titration) and indirect (excess KIO_3 and back titration with thiosulfate) methods. Lavine (66) uses an iodine titration to determine methionine as the periodide ($R_2S \cdot I_2$).

The use of iodate as a direct oxidant for sulfhydryl and disulfide groups has not been extensively studied although some literature is available. Williams and Driessen (133) employed iodate as well as dichromate and iodine in an attempt to determine the oxidation potential of cysteine by potentiometric titration. They state that apparently different products occur with the different reagents but do not try to establish these products. In an abstract which also mentions the reaction with iodine, Lavine (65) mentions that iodate in 1N HCl and in 1N H_2SO_4 oxidizes cysteine and cystine to cysteic acid. Some intermediates are also discussed. The reaction of cystine is said to be fast and that of cysteine somewhat slower. Williams and Wood (134) studied the reaction of iodate in 40% H_2SO_4 at 185° C. with various organic compounds including 16 amino acids, they state that only cystine, tryosine, and tryptophan react. In another type of study, Bauer (13) reported that a yellow color is produced when a protein is treated with iodate in aqueous solution. He states that only cysteine, cystine, and tryptophan react. In other studies on proteins, Weekers (132) reports that iodate oxidizes the sulfhydryl groups of beef lens,

altering the transparency; Goffart (44) states that iodate as well as some other oxidizing agents react reversibly with the sulfhydryl groups of the skin.

Several related works describe and discuss the "improver" action of iodate on wheat flour doughs (114); this action is thought to involve the oxidation of thiol groups (10). In connection with this, Hird and Yates (54) have studied the oxidations of cysteine, glutathione, and thioglycollate by iodate, bromate, and persulfate at pH 6. They indicate that cysteine is oxidized to cystine plus up to 10% higher products and that cystine is not oxidized. In another work, they studied the action of these reagents on certain proteins (55) related to wheat. Other studies on the effects of iodate on doughs are by Bloksma (15), Lee and Samuels (69), Meredith and Bashuk (85), and Tsen (124, 125). These authors study various aspects such as mixing, texture and related properties, and sulfhydryl content. One author (125) indicates some cleavage of S-S on oxidation.

Reaction of Insulin with Iodate

This section describes some of the pertinent properties and reactions of insulin. It is divided into three subsections which discuss general properties, oxidation, and reduction in that order.

General Properties

Insulin is a protein hormone obtained from the pancreas. Extensive reviews of the biological and chemical properties of insulin have been given by Jensen (57) and Archer (8).

Although active insulin preparations had been obtained earlier,

Abel (1) was the first to obtain crystalline insulin. The amino acid composition and sequence are well known and are reviewed by Tristram and Smith (123). The composition data come from the work of Harfenist (50). The amino acid sequence was studied by Sanger and coworkers (101, 102), who finally obtained the complete sequence for bovine insulin (97). The molecule (molecular weight 5734) consists of 51 amino acids arranged in two chains, 20 amino acids in the A chain and 31 amino acids in the B chain. The chains are joined by two disulfide bonds ("interchain") between the half cystine residues at A-7 and B-7 and at A-20 and B-19. A third disulfide ("intrachain") joins the half cystine residues at A-6 and A-11. The sequence of many insulins such as human, ox, pig, and so forth have been determined. They differ only in residues A-8, 9 and 10, i.e., those within the 6-11 disulfide ring. The conformation, secondary and tertiary structure, of this protein is not known.

Many reactions of the insulin molecule have been studied. The oxidation and the reduction of the disulfide group with possible changes to the organic chains will be discussed in subsequent subsections. Another reaction of interest is the iodination of tyrosin residues. These iodinations in insulin have been studied by Springell (113), Havinga (105), Scheraga (49), and others. Bruenfelt (19) gives the ultraviolet spectra of insulin at various levels of iodination. The spectrum changes because 3-iodotyrosine and 3,5-diiodo tyrosine absorb at higher wavelengths than tyrosine itself.

Oxidation of Disulfide Groups and Separation of Oxidized Chains

Jensen (58) reacted iodine with insulin and inactivated it. He

thought that the disulfide bonds had been oxidized. [Author's note: This was not proved and seems unlikely.] Sanger (99) oxidized insulin with performic acid and obtained derivatives of the two chains with the half cystine residues converted to cysteic acid residues. He succeeded in separating the chains (100) by dissolving the product of performic acid oxidation in 0.1M NH_4OH and adding 0.1M acetic acid until the pH was 6.5. The precipitate which forms was the B chain derivative. He then reduced the pH to 4.5 and discarded any precipitate. Finally, he evaporated the remaining solution to dryness obtaining the A chain derivative as the residue.

Two other methods of separation are described by Fittkau (39). One separation is accomplished by means of filtration through a Sephadex G-25 column; the A chain derivative emerges before the B chain derivative. The second method involves the use of a DEAE-Sephadex A-25 ion exchange column in 20% formic acid. The sample is dissolved in 20% formic acid and put on the column; the B chain derivative is eluted with more 20% formic acid while 1N HCl elutes the A chain derivative. [Author's note: This separation is probably due to the fact that the A chain derivative contains four sulfonic acid groups while the B chain derivative contains only two.]

Another method of separation and also of identification of these derivatives is electrophoresis. Two of the electrophoresis media generally employed for the separation of performic acid oxidized insulin derivatives are 20% formic acid (105,113) and pH 8.6 barbiturate buffer (128). In 20% formic acid, the A chain derivative moves toward the anode while the B chain derivative moves toward the cathode (105). In the barbituate buffer, both chains move toward the anode with the A

chain derivative moving faster (128). This separation is based on the total charge of the species.

Reduction of Disulfide Groups

This reduction has been studied by several authors with some conflicting results. Lindley (71) reported that lithium thioglycolate reduced only one disulfide except in the presence of urea where all three were reduced. He assumed this readily reduced disulfide to be the "interchain" bond. However, Cecil (24) stated that two disulfide bonds are reduced electrolytically while the third is not, and that the two bonds reduced are the "interchain" disulfides. He observed the same behavior in the reaction of insulin with sulfite (21) except under forcing reaction conditions. In another paper, Cecil (23) compares the reduction and sulfitolysis of several proteins including insulin and concludes that, in general, "interchain" bonds react more readily than "intrachain" bonds. Leach (68) studied the oxidative sulfitolysis of insulin, that is its reaction with sulfite in the presence of Cu^{++} ions and air, and found that all three disulfide bonds react. The reduction with 2-mercaptoethanol (92) also has been reported.

Another interesting reaction is that of lower molecular weight mercaptans, such as glutathione or cysteine, with insulin in the presence of glutathione-insulin transhydrogenose. This reaction gives a form of the A chain as one product (122). It has recently been shown (127) that the other product is a combination of A and B chains in a definite proportion, 1:4.

Another enzyme (32) of interest will catalyze the proper rearrangement of improperly joined disulfides is ribonuclease but will catalyze

the improper rearrangement of the disulfide bonds of insulin itself. This could indicate some instability in the arrangement of these bonds.

The oxidation of a mixture of reduced A and B chains of insulin has been studied to see to what extent the molecule and its biological activity can be regenerated (28,92,137). The combination of reduced synthetic chains by oxidation has also been studied (59,136). The results of both types of studies are quite controversial.

Reaction of Cysteine and Cystine with Periodate

In addition to the title subject, this section considers some historical aspects of the reactions of periodate with organic and biological compounds.

Since Malaprade (79) observed the ability of α-glycols to reduce periodate to iodate, many extensions and variations of this oxidation have been studied. A good review of some reactions of periodic acid and periodate with organic compounds is given by Jackson (56), while Dyer (37) has reviewed and discussed the biochemical aspects of periodate oxidations. Other reviews of this subject are available also (31).

Of more immediate interest to this work is the work of Nicolett and Shinn, who showed that α-amino alcohols would reduce periodate (67). They discuss the reaction of the β-hydroxy amino acid, serine:

$$\begin{array}{l} CH_2 - CH - COOH + IO_4^- \longrightarrow CH_2O + NH_3 + COOH + IO_3^- \\ \quad | \quad\quad | \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad | \\ \quad OH \quad\; NH_2 \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad CHO \end{array} \qquad \text{(II-1)}$$

The HOOC-CHO species may then react further according to the equation:

$$\begin{array}{l} COOH + IO_4^- \longrightarrow CO_2 + HCO_2H + IO_3^- \\ | \\ CHO \end{array} \qquad \text{(II-2)}$$

They indicate that tryptophan, methionine, and cystine are oxidized by periodate but give no details; they also mention that some of the other amino acids react very slowly. Periodate oxidation and determination of the amount of aldehyde released was developed into a method for the determination of serine (89) and threonine (106). The method was applied to the determination of these hydroxyamino acids in insulin hydrolysates (88).

Desnuelle (33,34,35) studied the action of periodate on certain proteins. In his first paper (33), he discusses the reaction of periodate with hydroxylysine, both free and bound in a protein. He states that a serine molecule with its amino group bound will not react with periodate. In another of these papers (35), he discusses the precipitation of egg albumin by reaction with periodate. He states that all the cysteine and cystine is consumed, 1/3 of the tryptophan and 1/10 of the tryosine; there is still more oxidation, presumed to be that of proline. Finally, he shows that the free amino acids cystine, tryptophan, methionine, proline, and tyrosine react with periodate at pH 5.5 and gives the extent of reaction for each in a specific interval.

Several other authors have also studied the effect of periodate on proteins. Maekawa and Kushibe studied the oxidation of ovalbumin (77) and lysozyme (78) by periodate. For ovalbumin they indicate the loss of cysteine and tryptophan with little effect on tyrosine, histidine, and cystine. With lysozyme, the primary study involved the loss of tryptophan which was related to the loss of activity. Grassman (47) studied the decomposition of collagen and procollagen at pH 8.6 which he thought involved mainly reaction of the periodate with the carbohydrate moieties of these substances. Another study by Zahn (138) using

collagen and keratin indicated oxidation of hydroxylysine; tyrosine, cystine, and methionine were also attached. Chatterjee (25) has studied the reaction with ovomucoid and ovalbumin and indicates that cysteine and cystine give cysteic acid, methionine gives the sulfone and sulfoxide, and tyrosine and tryptophan are eventually destroyed.

Another study is that by Lee (70) who treated several amino acids with periodate in 5N H_2SO_4 and found that many of them released ammonia in varying amounts. He makes no mention of the sulfur-containing amino acids. In another work, Clamp (29) studied all the α-amino acids at pH 2, pH 9 and in unbuffered solution. He indicated that all of these compounds are oxidized but at varying rates that increase with increasing pH. Cystine, cysteine, methionine, tryptophan, tyrosine, and histidine are oxidized even in polypeptide chains. These acids as well as serine, threonine, and proline are all rapidly and extensively oxidized when uncombined. The oxidation of the S-containing amino acids is said to occur mainly at the sulfur.

There are in general two titrimetric methods for determining the concentration of a periodate solution. The first, that of Fluery and Lange (40), involves the reduction of periodate to iodate with iodide at pH 8. The released iodine is then titrated with arsenite. The second method is that of Malaprade (80) and involves the reduction of the periodate to iodine with iodide in acid solution and the titration of the released iodine with thiosulfate.

CHAPTER III

CATALYSIS AND INHIBITION IN THE OXIDATION OF CYSTEINE AND OTHER MERCAPTANS BY FERRICYANIDE

This chapter reports only a few experimental details and results which were not included in the paper reproduced in Appendix I.

Experiments Using an Excess of Ferricyanide

The first experiments were done using an excess of ferricyanide. The buffer, pH 5.24, was prepared by dissolving 26.25 g of sodium acetate and 4.59 ml of glacial acetic acid in 2 liters of air-free water. The cysteine sample used is the same as sample I of the paper.

A series of EDTA solutions of concentrations from 1×10^{-4}M to 1×10^{-6}M was prepared in the buffer; also a 3×10^{-3}M cysteine solution and a 1.24×10^{-2}M ferricyanide solution were prepared in buffer. To 3 ml of buffer in a spectrophotometric cuvette, there was added 100µl of ferricyanide and the absorbance was measured at 410mµ. The reaction samples were prepared by mixing 9 ml of cysteine solution with 1 ml of buffer or of an EDTA solution, 3 ml of this was placed in the cuvette and 100µl of the ferricyanide solution was added; the final concentrations were 2.62×10^{-4}M cysteine, 3.95×10^{-4}M ferricyanide, and variable concentrations of EDTA. The decrease in absorbance at 410mµ with time was observed for 1 minute intervals over a 10 minute period.

One set of results is given in Table III-1. The value obtained for

TABLE III-1

EFFECT OF EDTA ON THE REACTION OF CYSTEINE WITH FERRICYANIDE

Cysteine, 2.62×10^{-4}M, and ferricyanide, 3.95×10^{-4}M; values in absorbance x 10^3.

Time (min)	0	0.74	0.96	1.93	3.87	5.81	7.74
1	167	160	155	295	350	374	389
2	151	155	158	248	343	382	379
3	150	156	155	200	337	375	379
4	150	157	156	173	328	-	369
5	152	159	156	164	333	365	376
7	-	-	-	132	320	367	-
10	154	156	154	142	315	360	378

the final absorbance with no added EDTA indicates a reduction in ferricyanide corresponding to only 92% of the cysteine taken. Much higher results, up to 98.0%, were obtained in other cases. These variations were possibly due to air oxidation of the cysteine solution. The procedure was therefore changed to that described in the Appendix. However, the inhibitory effect of EDTA is readily apparent in Table III-1.

Half-Reaction Time with Different Reagents

A set of experiments were done to ascertain the effect of pH on the reaction rate in solutions of the same EDTA concentration. It was desired that the medium should contain the same catalyst; to insure this as much as possible a pH 4.05 buffer was taken as the starting point; solid NaOH was added to a portion of this to make the pH 5.57; finally concentrated HCl was added to a portion of the pH 5.57 buffer to make the pH 5.28. The half reaction times at various EDTA concentrations for three such experiments are given in Table III-2. In the cases in which the rate could be measured, it increases as the pH is varied from 4.05 to 5.57, and increases again as the pH is lowered from 5.57 to 5.28. It is concluded that additional catalyst was added in the reagents used for the pH adjustment.

In another set of experiments, two ferricyanide samples were compared at pH 4. The results are given in Table III-3. The same buffer and cysteine were used in both cases but a different sample of ferricyanide was employed. It is obvious that the sample effects the results obtained.

TABLE III-2

EFFECT OF pH ON HALF REACTION TIMES

Cysteine, 4×10^{-3}M and ferricyanide, 4×10^{-4}M; time in minutes.

EDTA	pH		
$M \times 10^6$	4.05	5.57	5.28
3	<0.5	<0.5	<0.5
3.5	0.7	<0.5	<0.5
4	2.3	<0.5	<0.5
5	3.8	<0.5	<0.5
6	-	0.7	<0.5
7	>10.0	-	<0.5
8	-	1.9	0.5
9	-	3.2	1.1
10	-	-	2.9

TABLE III-3

HALF REACTION TIMES WITH TWO FERRICYANIDE SAMPLES

Cysteine, 4×10^{-3}M and ferricyanide, 4×10^{-4}M, time in minutes

EDTA $Mx10^6$	Ferricyanide Sample A	Ferricyanide Sample B
3	<0.5	<0.5
4.5	<0.5	1.1
6	1.2	-
7.5	1.7	5.6
9	3.8	7.8
10.5	6.4	9.0
12	>10	>10

Rates of Reaction of 3-Mercaptopropionic Acid and Octanethiol

In Appendix A, only a plot of the half life versus the EDTA concentration was given for 3-mercaptopropionic acid. A typical set of results for various EDTA concentrations is shown in Table III-4.

In Appendix A, only a plot of reaction rate versus EDTA concentration was given for 1-octanethiol. A typical set of results for various EDTA concentrations is given in Table III-5.

TABLE III-4

EFFECT OF EDTA ON THE 3-MERCAPTOPROPIONIC ACID OXIDATION

Values in absorbance x 10^3

Time (min)	EDTA, M x 10^6				
	0	1	2	3	4
0.5	245	275	381	385	386
1	179	238	354	384	385
2	112	190	331	364	380
3	84	160	320	348	378
4	64	129	280	341	374
5	49	108	259	334	365
10	24	46	151	239	337
15	9	20	90	151	324
20	0	9	37	82	291

TABLE III-5

EFFECT OF EDTA ON THE 1-OCTANETHIOL OXIDATION

Values in abosrbance x 10^3

Time (min)	EDTA, M x 10^4						
	0	1	2	4	6	8	10
0.5	310	341	359	365	368	366	367
1	257	296	323	330	354	353	365
2	190	231	278	283	334	350	360
3	133	182	241	260	325	339	356
4	100	145	205	225	-	326	349
5	75	114	176	196	298	320	343
10	22	36	93	100	241	288	313

CHAPTER IV

REACTION OF CYSTEINE AND CYSTINE WITH IODATE

Most of the experimental procedures, the results, and the conclusions, appear in the form of a paper suitable for publication which is reproduced in Appendix B. Only some details and pertinent observations not included in the paper will be given here.

Experimental

Materials and Solutions

A sample of cysteinesulfinic acid (I) from Calbiochem, A grade, lot 40075, was used in addition to that mentioned in Appendix B and designated (II) in this chapter. The reduced glutathione sample was from Schwarz Bioresearch, Inc., lot GL 6211.

The pH 2 buffer was prepared by dissolving 1.88 g of glycine and 1.46 g of NaCl in 25 ml of 1M HCl and diluting to 500 ml with water; this gives 0.05M glycine, 0.05M HCl, and an ionic strength of 0.10. The pH 3 buffer was prepared by dissolving 3.00 g of glycine and 2.34 g of NaCl in 10 ml of 1M HCl and diluting to 500 ml with water; this gives 0.08M glycine in 0.02M HCl at an ionic strength of 0.10.

Reaction Procedure

The reaction procedure given in Appendix B was usually followed. Two exceptions were sometimes made. In one variation, 100µl of a

5 x 10^{-3}M KI was added before addition of the KIO_3 solution. In the other variation, so-called "iodite-equivalent" solution was prepared by mixing 10 ml of 1.92 x 10^{-2}M KIO_3 with 10 ml of 0.96 x 10^{-2}M KI and 5 ml of the mixture replaced the usual 5 ml of KIO_3 solution; this mixture gave an overall oxidation state equivalent to 2.4 x 10^{-3}M KIO_2 in the reaction mixture.

Results and Discussion

Catalytic Effect of Iodide in 0.1M HCl

The results shown in Table IV-1 indicate that added iodide has a catalytic effect on the cystine-iodate reaction. This effect probably involves partial reduction of the iodate to a kinetically more reactive species, the nature of which cannot be determined from these results.

Reaction of Cysteine and Cystine at pH2

Cysteine, 1 x 10^{-3}M and 5 x 10^{-4}M, with 2.4 x 10^{-3}M iodate gave the results shown in Table IV-2. Cystine, 5 x 10^{-4}M, with 2.4 x 10^{-3}M iodate gave the results shown in Table IV-3. The reaction solutions were yellow. The reaction gave cysteic acid.

The cysteine reaction is clearly a two step process; this is discussed in Appendix B. The cystine reaction is clearly catalyzed by iodate. The rate of catalyzed reaction is almost linear with time to >80% reaction. Without iodide, the rate sensibly linear in the region, 10 to >80%. This strange behavior indicates a complex reaction mechanism.

TABLE IV-1

EFFECT OF IODIDE ON THE CYSTINE-IODATE REACTION

Cystine, 1 x 10^{-3}M, iodate, 2.4 x 10^{-3}M;

values in Q.

Time (min)	Iodide Added		
	0	$1x10^{-4}$M	$1x10^{-3}$M
5	2.27	3.74	6.01
10	6.17	7.42	8.84
15	8.58	8.98	9.84
30	10.00	10.02	9.96
60	10.03	10.03	10.05

TABLE IV-2

REACTION OF 2.4 x 10^{-3}M IODATE WITH CYSTEINE AT pH 2

Time (hrs)	Cysteine $1x10^{-3}$M	Cysteine $5x10^{-4}$M
0.1	3.56	3.61
0.25	3.69	3.93
0.5	3.78	4.10
1.0	3.98	4.13
3.0	4.57	4.72
6.0	5.14	5.16
24.0	5.97	6.18

TABLE IV-3

REACTION OF 5 x 10^{-3}M CYSTEINE WITH 2.4 x 10^{-3}M

IODATE AT pH 2

Time (hrs)	Iodide Added	
	None	1.67x10^{-5}M
0.25	----	0.49
0.50	0.77	1.53
1.0	2.74	3.98
1.5	5.44	6.53
2.0	7.75	8.47
2.5	9.17	9.54
3.0	9.67	9.77
6.0	10.04	9.96

Reaction of Cysteine and Cystine at pH 3

Cysteine, 1 x 10^{-3}M and 2 x 10^{-3}M, gave the results shown in Table IV-4. Cystine, 5 x 10^{-4}M, with 2.4 x 10^{-3}M iodate gave the results shown in Table IV-5.

The reaction solutions again were yellow. The product was cysteic acid.

The cysteine reaction is the usual two step process. The cystine reaction is strongly catalyzed by iodide ion.

Reaction of Cysteinesulfinic Acid at pH 0-2

The reaction of 1 x 10^{-3}M cysteinesulfinic acid (I) with 2.4 x 10^{-3}M iodate in 1M HCl gave a Q of 2.20 ± 0.01 in 5 minutes and this remained constant. This value if 10% greater than that required for oxidation to cysteic acid. A probable explanation is that the sample, nominally a dihydrate, had lost some water of crystallization. The neutralization equivalent of the sample, (I), was 174.2, in fair agreement with the molecular weight, 171.3; calculated on this basis, the Q value becomes 2.02.

The reaction of cysteinesulfinic acid (II), 1 x 10^{-3}M, with 2.4 x 10^{-3}M iodate at pH 2 gave a Q value of 1.75 in 5 minutes increasing to 1.91 in 15 minutes and remaining constant.

The product is cysteic acid.

Reaction of Cysteinesulfinic Acid at pH 3-4

The reaction of cysteinesulfinic acid, $CySO_2H$, with iodate at pH 4 gave rather erratic results which varied with reactant concentration. The reaction of 2.4 x 10^{-3}M $CySO_2H$ (I) with 2.4 x 10^{-3}M iodate gave a

TABLE IV-4

REACTION OF 2.4 x 10^{-3}M IODATE WITH CYSTEINE AT pH 3

Time (hrs)	Cysteine	
	$1x10^{-3}$M	$2x10^{-3}$M
0.1	3.91	3.84
0.25	4.30	4.60
0.5	4.75	4.95
1.0	5.11	5.22
3.0	5.61	5.53
6.0	5.79	5.73
12.0	6.10	6.03
24.0	6.11	6.25

TABLE IV-5

REACTION OF 2.4×10^{-3}M IODATE WITH 5×10^{-4}M CYSTINE AT pH 3

Time (hrs)	Iodide Added	
	None	1.67×10^{-5}M
0.5	0.09	0.10
1.0	0.13	0.20
3.0	0.13	0.47
6.0	0.11	0.81
12.0	0.43	2.88
24.0	3.31	7.53
48.0	10.29	10.20

Q of 0.55 in 5 minutes and this remained constant except for small flucuations. Addition of an equal volume of 2.4×10^{-3}M iodate to a sample of this (reducing $CySO_2H$ to 1.2×10^{-3}M and leaving iodate at 2.4×10^{-3}M) gave a Q of 0.74. The reaction of 4.8×10^{-3}M $CySO_2H$ (I) with 2.4×10^{-3}M iodate gave a Q of 0.39 after 5 minutes, and the value did not increase with time. Addition of an equal volume of 2.4×10^{-3}M iodate (giving 2.4×10^{-3}M $CySO_2H$ and 2.4×10^{-3}M iodate) gave a Q value of 0.56. There was no reaction between cysteic acid and "iodite-equivalent" solution (see Experimental section).

The reaction of 2.4×10^{-3}M "iodite-equivalent" solution with $CySO_2H$ (II), 1×10^{-3}M and 2×10^{-3}M, at pH 4 gave the results shown in Table IV-6.

The reaction of $CySO_2H$, 1×10^{-3}M and 2×10^{-3}M with 2.4×10^{-3}M iodate at pH 3 gave the results shown in Table IV-7. The reaction of $CySO_2H$ (II), 1×10^{-3}M and 2×10^{-3}M, with 2.4×10^{-3}M "iodite-equivalent" solution gave, Q values of 1.56 and 1.73 in 5 minutes, that increased to 1.82 and 1.90 in 6 hours, respectively.

The results obtained with cysteinesulfinic acid at pH 4 require some comment. A possible explanation is that there is a quasi-equilibrium established, possibly according to the equation:

$$CySO_2H + IO_3^- \rightleftharpoons CySO_3H + IO_2^- \qquad \text{(IV-1)}$$

The fact that dilution of the 4.8×10^{-3}M $CySO_2H$ reaction solution to 2.4×10^{-3}M gave the same Q values as the original 2.4×10^{-3}M $CySO_2H$ reaction solution supports this conclusion. Equilibrium constants of 0.133 for 1.2×10^{-3}M $CySO_2H$, 0.144 for 2.4×10^{-3}M $CySO_2H$, and 0.155 for 4.8×10^{-3}M $CySO_2H$, averaging 0.144 ± 0.006, are obtained on the

TABLE IV-6

REACTION OF 2.4×10^{-3} M "IODITE-EQUIVALENT"

SOLUTION WITH $CySO_2H$ AT pH 4

Time (hrs)	$CySO_2H$	
	1×10^{-3} M	2×10^{-3} M
0.1	0.62	0.53
0.25	0.60	0.54
0.5	0.75	0.59
1.0	0.79	0.66
2.0	1.02	0.80
3.0	1.38	1.02
6.0	1.93	1.53
12.0	2.02	1.94

TABLE IV-7

REACTION OF 2.4×10^{-3} M IODATE WITH $CySO_2H$ at pH 3

Time (hrs)	$CySO_2H$	
	1×10^{-3} M	2×10^{-3} M
0.1	0.56	0.43
0.25	0.56	0.48
0.5	0.47	0.34
1.0	0.36	0.40
3.0	0.59	0.38
6.0	0.77	1.80
24.0	1.81	1.87

basis of equation (IV-1) from the equilibrium constant expression:

$$K = [CySO_3H]\,[IO_2^-] \,/\, [CySO_2H]\,[IO_3^-]$$

Correction of the equilibrium constant values for the neutralization equivalent of the $CySO_2H$ sample (I) gives an average constant of 0.100 ± 0.005. If one assumes that hypoiodate, IO^-, is the product instead of IO_2^-, much worse agreement between the constants is obtained. Paper chromatography of the equilibrium solutions indicates the presence of both $CySO_2H$ and $CySO_3H$.

The $CySO_2H$ oxidation at pH 3 also indicates the establishemnt of a quasi-equilibrium but in this case complete oxidation to cysteic acid is achieved after some hours.

The use of "iodite-equivalent" solution as an oxidant at both pH 3 and 4 leads to complete oxidation to cysteic acid. At pH 4, the yellow "iodite-equivalent" solution became colorless upon mixing with $CySO_2H$ and remained colorless until the final value of Q was reached.

Reaction with Glutathione

Glutathione, 2.5×10^{-4}M, and 6×10^{-4}M iodate in 0.5M HCl gave $Q = 4.66 \pm 0.1$ in 30 minutes and $Q = 6.19 \pm 0.06$ in 24 hours. This oxidation follows the same pattern as that of cysteine itself in 0.1-1M HCl. The final value of Q = 6.19 is slightly high but indicates oxidation to the sulfonic acid derivative.

In acetate buffer of pH 4, the same concentrations gave $Q = 2.16 \pm 0.05$ in 30 minutes, which remained constant. The extent of reaction is much less than that for cysteine. The product indicated by the Q value is a mixture of oxidized glutathione and about 25% sulfonic acid

(there would, of course, be more of a less highly oxidized product).

At the same concentrations and pH 7, Q equalled 1.16 in 30 minutes, increased to 1.33 $\pm$ 0.1 in 3 hours, and then remained constant. This result is like that for cysteine with somewhat less higher products, 6 to 7% sulfonic acid. Oxidized glutathione was not studied.

Reaction with Other Amino Acids

The reaction of 1 x 10^{-3}M histidine, tyrosine, threonine, and serine with 2.4 x 10^{-3}M iodate in 0.5M HCl showed no reaction in 24 hours and only tyrosine appeared to show a very slight reaction (Q = 0.24) in 5 days.

These findings hold promise that the other amino acid residues present in proteins will not react with iodate. However tryptophan is attacked (41).

CHAPTER V

REACTION OF INSULIN WITH IODATE

Experimental

Materials and Solutions:

The insulin was crystalline bovine pancreas insulin, lots 55B-1850 and 74B-1020, from Sigma Chemical Company. Sephadex G-25 coarse, G-50 coarse and DEAE-Sephadex A-25 were obtained from Pharmacia. All other chemicals were of A.C.S.-reagent grade.

The water used in the preparation of solutions was obtained by condensation of steam and then passed through a bed of ion-exchange resin, Rexyn I-300 (H-OH), research grade (Fisher). The 1M HCl was prepared by diluting 41.5 ml of concentrated HCl to 500 ml with water; other solutions were prepared by diluting the 1M HCl. The starch indicator solution was 1% starch in 3% boric acid.

Iodate Oxidation Procedure:

The desired amount of insulin, usually 14.3 mg (to give a 10^{-4}M solution) was dissolved in 10 ml of 0.01M HCl and then mixed with 10 ml of 1M HCl; direct solution into 0.5M HCl was slow. Except in some cases, 100µl of a 5 x 10^{-3}M KI solution was added; finally, KIO_3 solution in 0.5M HCl was added, usually 5 ml of 4 x 10^{-3}M solution giving an 8-fold molar ratio of KIO_3 to insulin. The reaction mixture was allowed to

stand in a stoppered flask at room temperature. At intervals, 3 ml samples were removed and added to a quenching solution consisting of 5 ml of 0.5M KI, 3 ml of 1M HCl, and 1 ml of starch solution. The iodine released was then titrated to the disappearance of the starch-iodine color with 0.012M sodium thiosulfate solution.

The precipitate which appeared in the reaction solutions was removed either 30 minutes after precipitation and again 4 to 5 hours after precipitation or after several days. It was washed twice with acetone, twice with ether, and allowed to dry in air. Some samples including those used to check the weight of the precipitate were also dried under a reduced pressure in a vacuum desicator.

Performic Acid Oxidation Procedure

Performic acid was prepared by mixing 1 ml of 30% H_2O_2 with 9 ml of 90% formic acid. Five ml of this was then added to 10-50 mg of insulin. After 30 minutes, a two fold volume of acetone was added. The precipitate which formed was washed twice with acetone, twice with ether, and allowed to dry in air. This reaction was conducted at room temperature.

Amino Acid Analysis

The sample to be hydrolyzed, about 1μ mole in 0.5 ml of a suitable solvent (0.01M HCl for insulin and performic acid oxidized insulin, 0.01M NaOH for iodate oxidized insulin), was placed in a tube. To this was added 0.5 ml of 12M HCl giving a 6M HCl solution. The tube was connected to a vacuum system and the solution was frozen by inserting the end of the tube into an ethanol-dry ice mixture. The tube was sealed under a vacuum of 60-75μ and then placed in an oven at 110°C.

for 22 hours. After removal from the oven, the top of the tube was broken off and the contents were evaporated to dryness over solid NaOH in a vacuum desicator. These samples were analyzed with the Spinco Amino Acid Analyzer in the recommended manner (112).

Electrophoresis Procedure

The experiments were performed in a Gelman Rapid Electrophoresis Chamber with a Beckman Duostat Regulated Power Supply. Strips of Whatman No. 1 filter paper, 3/4 x 7 inch, were used. The sample, about 10µl containing about 0.7 mg of substance, was applied to the center of the dry strip. The media used were 20% formic acid and barbiturate buffer of pH 8.6 (4.41 g of sodium diethylbarbiturate, 2.34 g of sodium acetate, and 30.75 ml of 0.1M acetic acid in 1 liter of solution). The strips were dipped in the medium after application of the sample and stretched properly in the chamber (42). The chamber was covered and voltage then applied. The experiments were usually conducted at 200 volts for two 1 hour although some variations were tried. The strips were dried in air and then developed by dipping in a 0.5% ninhydrin solution in 95% ethanol.

Gel Filtration Procedure

A 1.5 x 25 cm column of Sephadex G-25 or G-50 was used. The solvent used was 0.05M $NaHCO_3$ with added NaCl to vary the ionic strength,

Ion-Exchange Chromatography Procedure

A 1 x 2 cm column was used (39), packed with DEAE-Sephadex A-25 ion-exchange resin which had been equilibrated with 20% formic acid.

The sample was dissolved in 3 ml of 20% formic acid and applied to the column; the eluate collected as the sample was applied was called fraction I. Elution was effected with 3 ml of 20% formic acid (fraction II) followed by 3 ml of additional 20% formic acid (fraction III). Further elution was effected by 4 ml of 1M HCl (fraction IV) followed by an additional 5 ml of 1M HCl (fraction V). The column was then re-equilibrated with 20% formic acid. The presence of protein components in the fractions was checked by measuring the absorbance at 276 mμ versus an eluant blank.

Precipitation Separation Procedure

This separation is based on that of Sanger (100) for the product of performic acid oxidation. The sample, 14 mg of oxidized material, was dissolved in 3 ml of 0.1M NH_4OH. To this was added enough 1M acetic acid to adjust the pH to about 5.3, about 0.28 ml. After 15 minutes, the precipitate was removed by centrifugation, washed twice with acetone, twice with ether, and dried in air. The supernatant solution was then adjusted to pH 4.5 with about 0.22 ml of 1N acetic acid. A slight addition precipitate formed that was removed and discarded. The resulting solution was then evaporated to dryness in a vacuum desicator over solid NaOH giving the second fraction.

Results

As mentioned in the experimental section, the reaction of insulin with iodate is accompanied by precipitation of the product. The consumption of iodate continues even after precipitation of the protein. The weight of the 30-minute precipitate is about 98% of that of the

initial material. The additional precipitate formed after 4 to 5 more hours is about 5% of the weight of the initial material. This recovery is probably less than that apparently indicated since the precipitates may not be completely dry.

The Q values, related to the extent of oxidation and obtained by measurement of supernatant iodate concentration when the 30-minute precipitate had been removed, averaged 18.3 ± 0.2 (14 samples). This value rose 19.6 ± 0.2 for these samples after 4 to 5 hours. It must be mentioned that the purity of the insulin sample is not known. Thus the absolute magnitude of these values may be slightly off but they are probably close. When the precipitate had not been removed, the Q value averaged 23.0 ± 0.1 (2 samples) after 6 hours and 26.4 ± 0.4 (6 samples) after 24 hours. All of these solutions contained added iodide. A plot of Q versus time for three reaction solutions, two of which contained added iodide and one of which did not, is shown in Figure V-1. The precipitate was not removed from any of these solutions.

In what follows, the term "iodate-oxidized insulin" will designate the precipitate that has been removed 30 minutes after precipitation. Other samples will be designated appropriately.

Figure V-2 shows the ultraviolet spectra obtained for a sample of unreacted insulin, a sample of iodate-oxidized insulin (Q = 18.3), and a precipitate which had been exposed to iodate for 4 days (Q = 29.6). The solvent is 0.05N $NaHCO_3$.

The results of the amino acid analysis of insulin, performic acid-oxidized insulin, and iodate-oxidized insulin along with the theoretical values for the amino acid composition are shown in Table V-1.

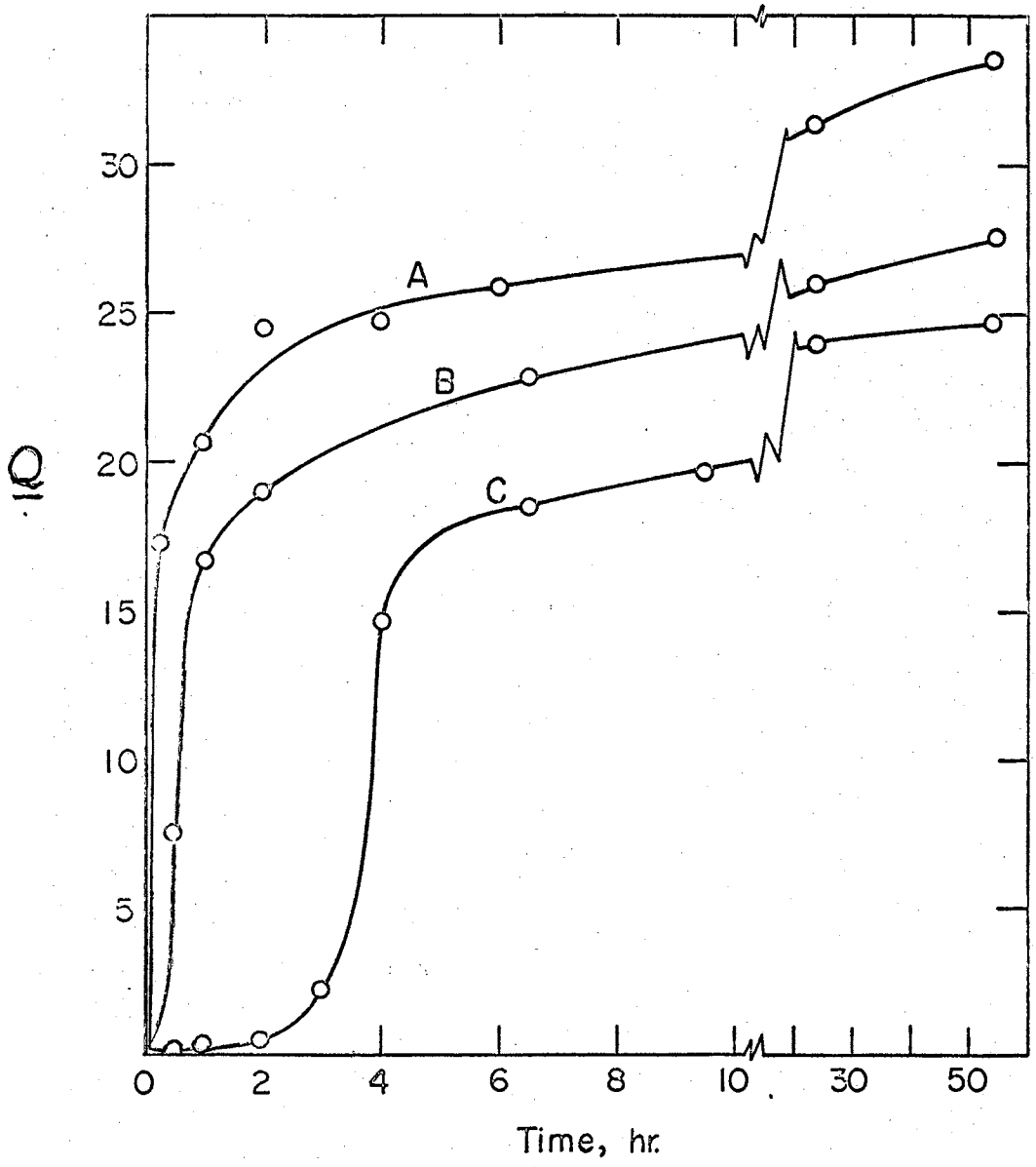


Figure V-1. Reaction of 1 x 10^{-4}M Insulin with 1.2 x 10^{-3}M Iodate. A - 1 x 10^{-3}M iodide, B - 1 x 10^{-4}M iodide, and C - no iodide.

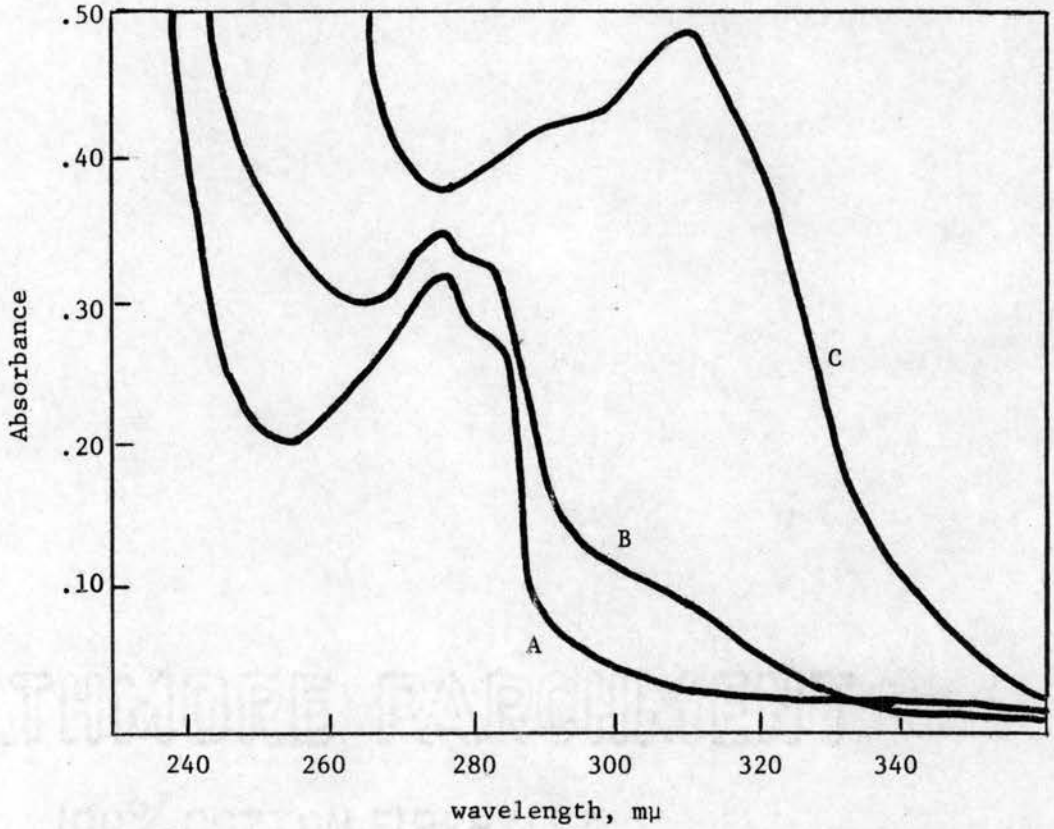


Figure V-2. The Ultraviolet Spectra of Insulins. The spectra shown are for: A - insulin itself, 7.30 mg/25 ml; B - 30-minute oxidized insulin, 7.60 mg/25 ml; and C - 4-day oxidized insulin, 7.60 mg/25 ml. The solvent is 0.05M $NaHCO_3$.

TABLE V-1

AMINO ACID ANALYSIS

As residues per molecule based on Lys = 1.00

Amino Acid	Insulin	Iodate Oxidized Insulin	Performic Oxidized Insulin	Theory
Lys	1.00	1.00	1.00	1
His	1.91	1.89	1.80	2
Amm	5.57	6.86	7.22	6
Arg	1.04	1.00	0.96	1
$CySO_3H$	----	1.71	4.73	0
AspOH	3.00	2.96	2.81	3
Thr	0.61	0.85	0.82	1
Ser	2.09	2.38	1.86	2
GluOH	7.22	7.12	7.01	7
Pro	1.13	1.18	1.17	1
Gly	4.30	4.30	4.24	5
Ala	3.22	3.48	3.07	3
½Cys	4.65	2.52	0.45	6
Val	4.74	4.12	4.19	5
Ileu	0.78	0.79	0.79	1
Leu	6.04	5.97	5.95	6
Tyr	3.83	3.09	3.18	4
Phe	2.87	2.86	2.76	3

The results of electrophoretic analysis in 20% formic acid and in barbiturate buffer are shown in Table V-2. The last sample cited is the product of the performic acid oxidation of an iodate-oxidized insulin sample.

The following additional electrophoretic experiments were performed in 20% formic acid. The iodate-oxidized insulin was subjected to the precipitation separation procedure. The pH 5.3 precipitate gave mainly the 0.9-1.4 cm band, while the residue contained primarily the 1.5-2.1 cm band. Performic acid oxidation of the pH 5.3 precipitate had only minor effects on its electrophoretic analysis. Oxidation of the other portion however shifted the band to the anodic side, approximately in the same position as the anodic band from performic acid-oxidized insulin. There was some decomposition observed in these samples. A 4-day iodate-oxidized sample showed essentially the same bands as iodate-oxidized insulin.

Gel filtration of iodate-oxidized insulin gave little or no separation into components. No better separation was obtained with performic acid-oxidized insulin.

The ion-exchange chromatography experiments on a performic acid-oxidized sample showed separation with one component appearing in the 20% formic acid fractions (I and II) and another component appearing in the 1M HCl fraction (IV). With iodate-oxidized insulin, the entire protein appeared in the 20% formic acid fractions (I and II). When unreacted insulin was tried, it also appeared in the 20% formic acid fractions (I and II).

TABLE V-2

ELECTROPHORESIS OF INSULIN SAMPLES

Position of band edges in cm from center of strip;

direction is (+) cathodic and (-) anodic.

Oxidation	Buffer	
	Formic Acid	Barbiturate Buffer
None	1.6-2.2 (+)	2.0-3.1 (-)
Performic Acid	0.9-1.4 (+) 0.8-1.3 (-)	0.6-1.5 (-) 4.2-5.2 (-)
Iodate	0.9-1.4 (+) 1.6-2.1 (+)	0.9-1.5 (-) 2.6-3.3 (-)
Iodate then Performic Acid	0.9-1.4 (+) 1.0-1.5 (-)	-----

Discussion

The Q value used to indicate the extent of reaction is equal to the number of equivalents of oxidant consumed or, in other words, the number of electrons transferred per molecule of insulin. In Appendix B, it is shown that the Q value for the oxidation of cystine to two cysteic acids is 10, to cystine disulfone ($CySO_2SO_2$ Cy) is 8, and so forth. The Q values obtained for the 30 minute iodate oxidation, approximately 18, could indicate either partial oxidation of all three disulfides or total oxidation of one and partial oxidation of one or both of the others. There is additional uncertainty since the purity of the insulin is not known. Oxidation of other amino acid residues is not expected since histidine, tyrosine, threonine and serine were shown not to react with iodate in Chapter V.

The shift in the ultraviolet absorption maxium from 276mμ for unreacted insulin to 310mμ for 4 day iodate-oxidized insulin indicates that the tyrosine residues are iodinated during the reaction. Comparison of these spectra with those obtained by Brunfeldt (19) indicates that the 30-minute iodate-oxidized precipitate might contain as much as 1 iodine atom per molecule of insulin while the 4-day sample contains 5 atoms of iodine or more per insulin molecule. (The pH of the medium is 0.5 units lower than that employed by Brunfeldt; it is not believed that this would make much difference.) If iodination occurs as follows:

$$InTyrH + I_2 \longrightarrow InTyrI + H^+ + I^-$$

It would lead to a Q value of 2 per iodine atom added. Thus the Q due to oxidation is 16 to 17 for the 30-minute iodate-oxidized sample. On this basis even for the 4-day sample, the Q value due to oxidation

would be less than 20. These results show that the initial rapid consumption of iodate shown in Figure V-1, which causes precipitation, is primarily oxidation. The slow heterogeneous reaction of the precipitate upon standing in the solution, on the other hand, is primarily iodination of tyrosine residues.

The results for the amino acid analysis of iodate-oxidized insulin indicate two cysteic acid residues per molecule. They also indicate more than two half-cystine residues per molecule. For insulin itself, the number of half-cystine residues is low, 4.65 rather than 6, due to some destruction on hydrolysis. There are probably 3 or even more half-cystine residues per molecule of iodate-oxidized insulin. For the other amino acids, there is fairly good agreement between the three samples and with the theoretical values. This again indicates no oxidation of the other amino acids by iodate.

Both formic acid and barbiturate buffer electrophoresis indicate that there are two separate components in the iodate-oxidized insulin. The results in formic acid show the same bands as were previously reported (105) for performic acid oxidized insulin; the cathodic band is the B chain derivative while the anodic band is the A chain derivative. Of the two fractions produced by iodate oxidation, one appears to be the B chain derivative while the other migrates even farther toward the cathode. In barbiturate buffer, Voelker (128) reports that the A and B chain derivatives from performic acid oxidation both move toward the anode with the A chain derivative moving faster. The results obtained in this work for iodate-oxidized insulin, show a fraction which appears to be the B chain derivative and a fraction which does not move as far toward the anode as the A chain derivative. Such behavior,

as that in formic acid, would be characteristic of an A chain derivative with less than 4 sulfonic acid groups, either none or possibly two. The fractions obtained by fractional precipitation contain primarily one or the other of the bands and thus confirm that the insulin molecule is split by the oxidation. The apparent lack of effect of oxidation by performic acid on the pH 5.3 precipitate, normally the B chain derivative as well as the position of this fraction further substantiates the conclusion that one product is the usual performic acid-oxidized B chain derivative. Since performic acid oxidation of the other fraction gives primarily a product which behaves like the performic acid-oxidized A chain derivative, the conclusion that the second product is a modified A chain derivative is further supported.

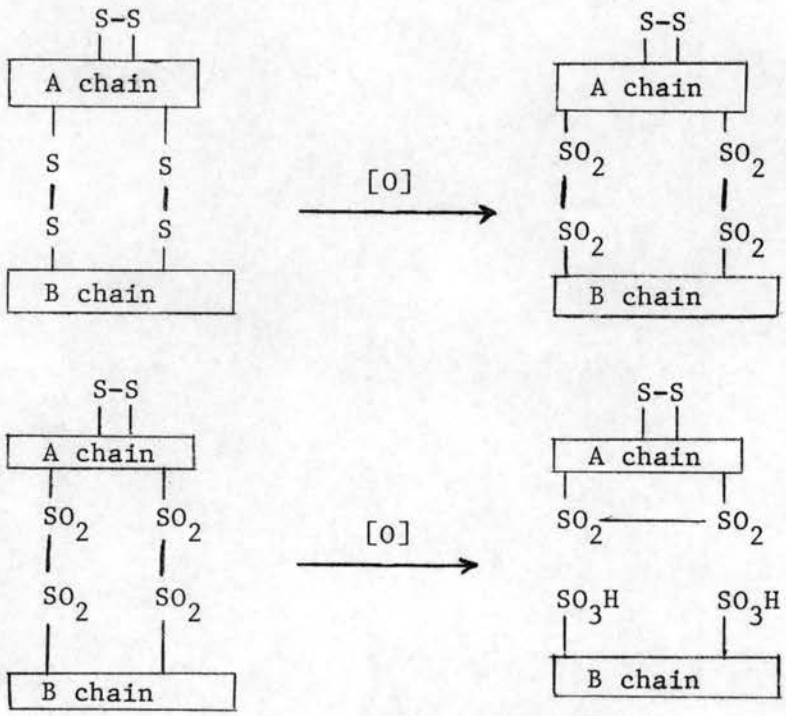
No conclusion may be drawn from the gel filtration experiments since neither iodate- nor performic acid-oxidized insulin is adequately separated.

According to Fittkau (39), the fraction of performic acid-oxidized insulin eluted by 20% formic acid from a Sephadex A-25 ion exchange column is the B chain derivative while the more acidic A chain derivative is eluted with the 1N HCl. Although all the protein is eluted by 20% formic acid for iodate-oxidized insulin, this would be expected if an A chain derivative with less sulfonic acid groups were present since the separation of the performic acid-oxidized sample appears to be dependent on the number of sulfonic acid groups in the species. Thus an A chain derivative with no or even two sulfonic acid groups would probably behave more like a B chain derivative or insulin itself.

A possible preference of the oxidation for the "interchain" disulfides over the "intrachain" bond would be somewhat expected from

the results of Cecil and Loening (21) and of Cecil and Weitzman (24) mentioned earlier. In both cases, enhanced reactivity of the "interchain" disulfide over the "intrachain" disulfide is attributed to the stability of the intrachain ring and to steric effects. These same effects might just as readily hinder the oxidation of the "intrachain disulfide", at least by milder oxidants such as iodate as compared to performic acid, for example.

The following facts concerning the iodate oxidation are then indicated: (1) the extent is somewhat less than that for two disulfides; (2) there are apparently two sulfonic acid groups produced; (3) one product appears to be the usual B chain derivative; (4) the other product appears to be a modification of the usual A chain derivative but containing a reduced sulfonic acid content, probably none, and (5) iodination of the tyrosine residues occurs to some extent. One possible explanation for the above facts is shown in the following reactions:



Although such a process might involve some refolding of the A chain, this would give 18 equivalents consumed by oxidation and would produce a normal B chain derivative and an A chain derivative having no sulfonic acid groups which should behave much as discussed above. This is of course not the only possible reaction, however it is in keeping with the observed results.

CHAPTER VI

REACTION OF CYSTEINE AND CYSTINE WITH PERIODATE

Experimental

Materials:

L-Cysteine hydrochloride·H_2O, B grade, was from Calbiochem; L-cystine, reagent grade, from Fisher Scientific Company; L-cysteic acid, CFP, from Calbiochem,; Glutathione, lot GL6211, from Schwarz Bioresearch; Oxidized glutathione, Kontrol-Nr. 6262319 from C. F. Boehringer and Soehne, Germany; sodium meta-periodate, reagent grade, lot 25445, from Baker Chemical Co. All other chemicals were of A.C.S. reagent grade. Water used in this work was obtained by condensation of steam, then passed through a column of Rexyn IRG-501 from Fisher Scientific Co.

The 1M HCl solution was prepared by placing 41.5 ml of concentrated HCl in a 500 ml volumetric flask and diluting to volume with water; other HCl solutions were prepared by diluting this solution.

The buffers used in this work were prepared in general according to Britton (18) with some variations. The acetate buffer of pH 1.09 was prepared by dissolving 16.41 g of solium acetate in 23.24 ml of concentrated HCl and diluting this to 1 liter, giving a solution 0.20M in acetic acid and 0.08M in HCl. The pH 4 acetate buffer was prepared by dissolving 8.20 g of sodium acetate in 6.64 ml of concentrated HCl

and diluting to 500 ml with water, giving a solution 0.16M in acetic acid and 0.04M in sodium acetate. The pH 7 phosphate buffer was prepared by dissolving 3.68 g of $NaH_2PO_4 \cdot H_2O$ and 5.68 g of Na_2HPO_4 in 1 liter of water giving a solution 0.0267M in NaH_2PO_4 and 0.0400M in Na_2HPO_4.

The starch indicator solution (37) prepared by adding 1 g of soluble starch in 5 ml of cold water to 3 g of boric acid in 100 ml of boiling water; this solution was boiled for 10 additional minutes, cooled and stored. The 80% phenol solution used in the paper chromatography work was prepared by dissolving 80 g of phenol in 20 g of water; this solution was stored in a glass stoppered bottle under a thin layer of petroleum ether. The ninhydrin solution used in this work was prepared by dissolving 0.1 g of ninhydrin and 0.405 ml of Symm-collidine in 25 ml of 95% ethanol.

Equipment:

A Beckmann Model G pH meter was used in checking and adjusting buffer and solution pH values. The acid-base titrations were performed using the Radiometer Titrator and Titrigraph.

Reactions and Sampling:

The reactions were conducted in glass stoppered flasks at room temperature, 20-4°. A sample of cysteine, cystine, cysteic acid, or glutathione, was weighed out, and dissolved to give 100 ml of solution. To make the cystine solutions of pH 4 and 7, the sample was dissolved in 1 ml of 1M HCl, some buffer was added, 1 ml of 1N NaOH was added, and the solution was diluted to volume with buffer. To 25 ml of the

solution was added 25 ml of $NaIO_4$ solution of the desired concentration. In some cases, one solution was poured into the other; in other cases, the periodate solution was added dropwise with vigorous stirring. The start of the $NaIO_4$ addition was taken as the start of the reaction.

At various time intervals after the start of the reaction, the solution was sampled. This was done by adding a 3 ml aliquot of the solution to a quenching solution, which consisted of 5 ml of 1M KI, 3 ml of 1N HCl, and some water. The iodine produced was then titrated with a standard thiosulfate solution; 1 ml of starch indicator was added as the endpoint was approached.

A blank solution was also prepared in all experiments. This solution consisted of 25 ml of 0.1N HCl solution or buffer and 25 ml of the $NaIO_4$ solution. This solution was sampled in the same manner as the reaction solution.

The sodium thiosulfate solution was usually standardized prior to each experiment. This was done by adding an aliquot of a freshly prepared potassium iodate solution to the quenching solution. The iodine released was then titrated with the standard thiosulfate solution.

Paper Chromatography Procedure:

The paper chromatography was performed on 2 x 13 cm strips of Whatman No. 1 chromatography paper. The moving phase was 80% phenol in water. The strips were developed with ninhydrin solution.

The procedure used in these experiments was essentially that of Fieser (38). Two lines are drawn across the strip 1.5 cm from each end, i.e. 10 cm apart. A small spot of sample is then placed at two points

on the starting line. The strip is inserted into a large test tube with 0.3 ml of 80% phenol in the bottom. This tube is stoppered and allowed to stand until the phenol front crossed the finish line. The strip is removed from the tube, sprayed with acetone to remove the phenol, and allowed to dry. After drying, the strip is sprayed with the ninhydrin solution and again allowed to dry while the color develops.

The distance of the center of a spot from the starting line is measured; this is divided by 10 cm, the distance the phenol travels, and the result is the R_f value for the component forming the spot.

Determination of the Hydrogen Ion Released by the Reaction:

These determinations were made by titration with sodium hydroxide solution. The titration curves were directly obtained.

The first step in this procedure was to prepare 5 ml of a 0.03M cystine solution in 1N HCl and to dilute this to 50 ml. Ten ml of this was placed in a stoppered flask and 5 ml of 0.1M sodium periodate was added. In a second flask, 10 ml of the cystine solution was placed and 5 ml of water was added to this solution giving a blank for checking the initial hydrogen ion concentration. After 2 hours, a 1 ml sample of the reaction solution was placed in the Titrimeter beaker, diluted slightly with water, and titrated automatically with 0.0884N NaOH with the titration curve being recorded. A blank sample was treated in the same manner. These titrations were then repeated after five days.

Results

The reactions of periodate with cysteine in various conditions will be discussed first. For fast addition, in pH 1.09 buffer, with

5×10^{-3}M cysteine and 1×10^{-2}M periodate, the reaction gave a value above 6 for Q, a measure of the extent of reaction, in less than one minute. This value then varied erratically for the duration of the experiment. The average Q after 24 hours for 4 runs was 6.9 ± 0.07. For slow addition, in 0.05N HCl, with 5×10^{-3}M cysteine and 5×10^{-2}M periodate, the reaction gave Q values above 7 in 15 minutes. This value fell with time, giving an average of 7.4 ± 0.07 after 1 hour and 6.38 ± 0.07 after 6 hours. The reaction of 5×10^{-3}M cysteine and 5×10^{-2}M periodate in pH 4 acetate buffer was also very rapid, giving an average value of 5.34 ± 0.6 (for 3 samples) after 15 minutes. This value rose to a maximum of slightly over 7 in less than an hour, then dropped to about 6.9 in the second hour and finally rose again. The value after 24 hours was 7.64 ± 0.08 (3 samples). The reaction of 2.5×10^{-3}M cysteine with 2×10^{-2}M periodate in pH 7 phosphate buffer was slightly slower giving 4.40 ± 0.07 after 30 minutes and rising fairly steadily to 8.07 ± 0.02 in 24 hours (6 samples) and to 8.94 ± 0.04 in 96 hours (for 4 of the aforementioned samples).

The result for cystine was in general more reproducible and much less erratic. In pH 1.09 buffer with 2.5×10^{-3}M cystine and 5×10^{-2}M periodate, the Q value rose above 9 in two hours and to 10.02 ± 0.01 in 24 hours. In 0.05N HCl, the reaction again proceeded steadily; it reached completion in 1 to 2 hours depending on concentration and gave a final Q value of 10.00 ± 0.04. A typical result for Q versus time for this reaction and that of cysteine in the same medium is given in Table IV-1. For 2×10^{-4}M cystine and 5×10^{-4}M periodate in pH 4 buffer, the reaction was very slow; Q was 1.93 ± 0.10 in 1 day, 4.70 ± 0.03 in 5 days, and 4.96 ± 0.03 in 7 days. For 2×10^{-4}M cystine and 5×10^{-4}M

TABLE VI-1

REACTION OF 5 x 10^{-2}M PERIODATE WITH 5 x 10^{-3}M

CYSTEINE OR CYSTINE

Time (hrs)	Amino Acid	
	Cysteine	Cystine
0.5	7.21	0.15
0.75	----	0.86
1.0	----	2.46
1.25	----	3.60
1.5	7.07	5.57
1.75	----	7.50
2.0	6.90	8.93
2.5	----	9.91
3.0	6.60	9.95
4.0	6.27	----
5.0	6.15	----
6.0	6.29	9.98
9.0	6.50	----
13.0	6.61	----
24.0	6.63	----

periodate in pH 7 buffer, the reaction was quite fast, giving Q = 5.61 $\pm$ 0.04 in 30 minutes and remaining constant at 5.95 $\pm$ 0.01 after about 2 hours.

The attempted oxidation of cysteic acid with periodate in 0.05N HCl showed that no reaction occurs in 24 hours.

The reaction of 2.5 x 10^{-4}M reduced glutathione (glSH) with 5 5 x 10^{-4}M periodate in 1N HCl gave an Q value of slightly over 4 in 30 minutes and increased to a value of 5.51 $\pm$ 0.09 after 24 hours and 6.05 $\pm$ 0.09 after 72 hours (2 samples). Two samples of the reaction of 2.5 x 10^{-4}M glSH and 5 x 10^{-4}M periodate in 0.5 N CHl gave slightly under 4 in 30 minutes and increased to 4.83 $\pm$ 0.05 in 24 hours. The same concentrations of reactants in pH 4 buffer gave 3.67 $\pm$ 0.01 after 30 minutes and increased to 5.25 $\pm$ 0.1 after 24 hours. The same concentrations in pH 7 buffer gave 2.85 $\pm$ 0.02 in 30 minutes and increased to 4.28 $\pm$ 0.01 in 24 hours. None of these samples showed the erratic behavior of the cysteine samples. Two samples of oxidized glutathione (glSSgl), 2.5 x 10^{-4}M were oxidized with 5 x 10^{-4}M periodate in 0.5N HCl giving 3.03 $\pm$ 0.02 in 30 minutes and increasing to 6.43 $\pm$ 0.08 in 6 hours.

Paper chromatography of the product obtained from cystine and periodate in 0.05M HCl gave a spot of R_f = 0.10; cysteic acid has the same R_f value; for cystine, R_f = 0.24. No paper chromatography was performed on the other reactions.

The titration curves with base for cystine gave two breaks, one for titration of the carboxyl groups and excess HCl and a poor one for titration of the NH_3^+ group. Titration of a cysteic acid solution also gave two breaks, one for titration of the carboxyl group, sulfonic

acid group, and excess HCl and a second fair one for the NH_3^+ group. Titration of the reaction solution gave only one break corresponding apparently to titration of the carboxyl groups, product acid groups and excess HCl. No break for titration of the NH_3^+ group was discernible. By subtracting the amount of base required for the initial break for the cystine solution from that required for the same initial cystine concentration after reaction with periodate, the amount of H^+ ions released in the reaction could be calculated. This was 0.0085 mmoles of H^+ per 0.01 mmole of cystine after 2 hours reaction of a 5×10^{-3}M cystine solution with 5×10^{-2}M periodate in 0.05N HCl. The same solution after standing 5 days showed an increase of 0.02 mmoles of H^+ per 0.01 mole of cystine.

All of the reactions solutions of cysteine and cystine were yellow-orange in color but tended to fade with time. Some of them contained a black precipitate in the bottom. Extraction of some of these reaction solutions with carbon tetrachloride gave a pink coloration to the organic phase.

Discussion

Before discussing the nature of the products derived from the amino acids, some consideration will be given to reduction products derived from periodate. Iodate is a possible product, commonly obtained in neutral medium. However, reduction must go beyond that stage in at least some of the cases studied in this work. The yellow color observed in aqueous solution, which gave a pink color upon extraction into carbon tetrachloride indicates the presence of free iodine; the black precipitate obtained in some concentrated reaction solutions must have

been solid iodine. It may be shown in the same manner as was done for iodate in Appendix B that the nature of this product will not affect the calculations.

The Q value corresponds to the number of electrons per molecule of mercaptan or disulfide reactant lost in going to product. Therefore the expected Q values for certain products are the same as those with iodate and are discussed in Appendix B.

The oxidation of cysteine was rapid but inconsistent Q values were obtained. The final Q values of about 7 at pH 1.09, 6.4 in 0.05N HCl, 7.6 in pH 4 acetate buffer, and 9 in pH 7 phosphate buffer, are all above the value of six required for oxidation to cysteic acid. Since cysteic acid itself is not oxidized by periodate, at least in 0.05N HCl, and presumably in the other media also, these high values cannot then be explained by assuming further oxidation of the cysteic acid. Thus we must look for some other entirely different oxidation sequence.

One possibility is obtained by analogy with the oxygen analog of cysteine, serine; it reacts with periodate according to reactions (II-1) and (II-2) on page 14. An analogous partial reaction for cysteine would be:

$$\begin{array}{l} CH_2 - CH - COOH \\ | \quad\quad | \\ SH \quad NH_2 \end{array} + H_2O \longrightarrow CH_2S + NH_3 + \begin{array}{l} COOH \\ | \\ CHO \end{array} + 2H^+ + 2\,e \qquad \text{(VI-1)}$$

followed by:

$$\begin{array}{l} COOH \\ | \\ CHO \end{array} + H_2O \longrightarrow CO_2 + HCO_2H + 2H^+ + 2\,e \qquad \text{(VI-2)}$$

These two reactions would lead to a Q of 4. However the CH_2S species from (VI-1) might be expected to react further. Two of possible half reactions are:

$$CH_2S + 5\ H_2O \longrightarrow CH_2O + SO_4^{=} + 10\ H^+ + 8\ e \qquad \text{(VI-3)}$$

or

$$CH_2S + 3\ H_2O \longrightarrow CH_3SO_3H + 4\ H^+ + 4\ e \qquad \text{(VI-4)}$$

Although these reactions for thioformaldehyde are not discussed as such, Reid (95) reports an oxidation analogous to (VI-3) for thiobenzophenone that gives the oxygen analog and the sulfate ion. A reaction analogous to (VI-4) is also discussed by Reid (96) for thiourea. From reactions (VI-1), (VI-2), and (VI-3), one gets the sum Q = 12; from (VI-1), (VI-2), and (VI-4), Q = 8. It is believed that the oxidation of cysteine by periodate procedes, at least in part, by splitting the carbon-sulfur bond, which would be analogous to the periodate oxidation of serine. It is possible that some oxidation to cysteic acid also occurs.

Although the reaction as indicated by the 15 to 30 minute Q values is probably somewhat slower at higher pH, the extent of the reaction appears to increase with increasing pH. This effect is in agreement with Clamp (29) and may possibly be connected with the oxidation of the intermediates produced in the initial reaction. No explanation for the somewhat irratic behavior of the results is evident, although the reaction intermediates may play some part in this effect also.

The value of 10 obtained for Q in the oxidation of cystine both at pH 1.09 and in 0.05N HCl indicates oxidation to cysteic acid according to Appendix B. The results of the paper chromatograms also definitely indicate cysteic acid as the product in 0.05N HCl. The production of 2 moles of H^+ per mole of cystine oxidized according to the titration with base agrees with the expected reaction if iodate is the reduction product. This may be seen from the reaction:

$$H_2O + CySSCy + 5\ IO_4^- \longrightarrow 2\ CySO_3^- + 5\ IO_3^- + 2\ H^+ \qquad \text{(VI-5)}$$

The lower value obtained earlier where free iodine was in the solution is also to be expected, since for reduction of periodate to I_2, the reaction is:

$$2\ H_2O + 7\ CySSCy + IO_4^- \longrightarrow 14\ CySO_3^- + 5\ I_2 + 4\ H^+ \quad \text{(VI-6)}$$

This gives 4/7 mole of H^+ ion per mole of cystine which is 0.57 or less than the observed value of 0.85. However reaction of the iodine with excess periodate will produce the additional 10/7 of a mole of H^+ necessary to give to observed final ratio 2 moles of H^+ per mole of cystine.

As stated in the results the reaction of cystine at pH 4 is very slow apparently being incomplete even after 7 days. The reaction in pH 7 buffer is surprisingly fast indicating a different pH effect than between pH 1 and 4. A final Q value of 6.0 is obtained which would correspond to the formation of cysteinesulfinic acid or of cystine sulfone sulfoxide, if only one product were present; however a mixture of products may be produced.

The more consistent results obtained with glSH also indicate a different reaction than with cysteine. The value of 6.0 obtained for this reaction in 1N HCl indicates the expected oxidation to the sulfonic acid derivative. The values obtained in the other media can be due to incomplete oxidation to the sulfonic acid. The reaction rate decreases as the pH increases. None of these samples show the overoxidation observed with cysteine. Periodate does not cleave serine derivatives in which the NH_2 group is tied up such as by an amide linkage (33). By analogy, the same behavior would be expected for cysteine where its amino group is tied up. This is the case in glutathione where this amino group forms an amide linkage from the cysteine to a glycine

molecule.

In conclusion it appears that although the reaction of cysteine itself is very complex, the reaction of glutathione, in which the amino group is bound, gives cysteic acid, as does the oxidation of cystine in acid solution. Although these reactions hold some promise for protein studies, the iodate oxidation discussed in Chapters III and IV is more promising.

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APPENDIX A

INHIBITION AND CATALYSIS IN THE OXIDATION OF CYSTEINE AND OTHER MERCAPTANS BY FERRICYANIDE

ABSTRACT

The oxidation of cysteine by ferricyanide in aqueous solution is strongly inhibited by ethylenedinitrilotetraacetate in a manner which indicates that the reaction is catalyzed by traces of metal ions usually present as impurities, probably iron and/or copper. Similar effects have been demonstrated in the reaction of ferricyanide with 3-mercaptopropionic acid and 1-octanethiol. Representative rate measurements are reported.

INTRODUCTION

Mercapto compounds usually react readily with oxygen or other oxidizing agents (115) and the reactions have important applications, especially in living organisms (12). The reaction of ferricyanide with cysteine in neutral medium has been utilized for the quantitative determination of the latter substance (82,6,129). In one of the investigations of this reaction, it was noted that copper(II) ions catalyze the reaction and that cyanide inhibits it, and it was suggested that the inhibition is due to reaction of the cyanide with catalytic impurities present in the reagents (6).

The probable role of catalysts in this reaction was brought to the attention of the writers when they added ethylenedinitrilotetraacetate

[as the disodium salt, $NaH(O_2C)_2NCH_2CH_2N(CO_2)_2HNa$, henceforth symbolized by EDTA] to solutions of cysteine in order to protect them from air oxidation. It was found that, in addition to the effect desired, EDTA strongly inhibited the reaction with ferricyanide as well. The present paper reports an investigation of this phenomenon.

Recently, the kinetics of the reactions of ferricyanide with 3-mercaptopropionic acid (16) and with 1-octanethiol (62) have been investigated. In the former case, the possibility of catalysis by traces of metal ions was not specifically considered, while in the latter case the inhibitory effect of cyanide was explained by a mechanism that did not involve metal-ion catalysis. In the present work, the behavior of the above-named mercaptans was reinvestigated to determine whether such catalysis was in fact involved.

METHODS

Materials. Cysteine hydrochloride monohydrate was obtained from the California Corp. for Biochemical Research, Los Angeles (sample I, B grade; sample II, A grade); 3-mercaptopropionic acid from Evans Chemetics, Watertown, New York (label purity, 99.2%); 1-octanethiol from Matheson, Coleman and Bell, Cincinnati. Potassium ferricyanide was of ACS reagent grade; sample I was obtained from Fisher Scientific Company, St. Louis, and sample II from Baker and Adamson, New York. Other chemicals were also ACS reagent grade, except acetone, which was of CP grade.

The water used in the experiments was obtained by condensation of steam and passed through a bed of ion-exchange resin, Rexyn IRG-501, reagent grade (Fisher Scientific Co.). The water was then boiled,

cooled slowly with a stream of nitrogen bubbling through, and stored under nitrogen. The nitrogen was of commercial grade. It was passed through a vanadium(II) solution to remove oxygen (84).

Acetate buffer, pH 4.05, was prepared by mixing 9.18 ml of glacial acetic acid and 3.282 g of sodium acetate in deaerated water to give 1 liter; acetate buffer, pH 5.57, was prepared from pH-4 buffer by adding 5.600 g of sodium hydroxide per liter. Carbonate buffer, pH 9.80, was prepared by dissolving 2.103 g of sodium bicarbonate, 2.650 g of sodium carbonate, and 0.5845 g of sodium chloride in deaerated water to give 1 liter. These buffers were stored under nitrogen.

Unless otherwise specified, the solutions of cysteine and of ferricyanide were used within 6 hrs from the time of preparation.

Apparatus. Spectrophotometric measurements were made with a Beckman DU spectrophotometer using 1-cm silica cells. Measurements of pH were made with a Beckman Model G meter, standardized with commercial buffers.

Experimental Procedure. Weighed amounts of cysteine hydrochloride or of 3-mercaptopropionic acid were dissolved in acetate buffer; 1-octanethiol was dissolved in 30 ml of carbonate buffer and this diluted with acetone to 100 ml. Solutions of EDTA and ferricyanide were prepared in the same buffer as the mercaptan and the reagents were mixed to give the following concentrations at zero time: mercaptan, 0.0038 - 0.004 M; ferricyanide, 4.0×10^{-4} M; EDTA, 0 to 3×10^{-5} M, as designated.

To measure the rate of reactions which would be complete in a few minutes, the reagents were first brought to $25^{\circ} \pm 0.1^{\circ}$ and then mixed in a spectrophotometer cell; the absorbance was measured at appropriate

intervals of time, without attempting to control the temperature of the cell. In experiments extending for longer periods of time, larger amounts of reagents were mixed and kept in the thermostat and absorbance measurements were done on aliquot portions that were withdrawn and placed in the spectrophotometer cell at the appropriate time.

Ferricyanide has a strong absorption maximum at 418 mμ (17,16) while the other reagents do not absorb at this wavelength. The progress of the reaction could therefore be conveniently followed by spectrophotometric measurements. The measurements were actually made at 410 mμ, and the molar absorbancy index of ferricyanide was taken as 990.

RESULTS

In acetate buffer of pH 4.0 with 3.8×10^{-3} M cysteine and 4×10^{-4} M ferricyanide, the reaction was essentially complete in less than 1 min and no measurements of rate were made. When EDTA was added to the reaction mixture, the rate was reduced, as exemplified by the curves in Fig. 1. In the range of EDTA concentrations that gave conveniently measurable rates, reproducible results could be obtained with freshly prepared solutions made up from the same samples of solid reagents and the same batch of distilled water, but quite different rates might be observed with different samples (for purposes of identification, these will be denoted by Roman numerals, sequentially assigned). With a particular sample of cysteine (I) and ferricyanide (I) made up in acetate buffer (I) in the presence of 9×10^{-6} M EDTA, for example, the average half-reaction time was found to be 3.5 min with an average deviation of $\pm$ 0.3 min; with the same reagents made up in acetate buffer (II), the half-time was 13.6 min; with everything the same as in

the first instance except a different sample of ferricyanide (II), the half-time was 7.7 min. The age of the ferricyanide solutions was an additional variable, the same solution giving a faster rate after standing 1-2 days; in general, freshly prepared solutions were used, so this effect did not come into play. Cysteine samples (I) and (II) reacted at nearly the same rate, but this may not be the case generally.

Figure 2 shows the results obtained in a different type of experiment, in which the ferricyanide remaining after a short reaction period, 45 sec, was measured in the presence of increasing EDTA concentration (cysteine, I; ferricyanide, I; acetate buffer, III). It can be seen that a plot of the data has an inflection point at about 1.9×10^{-6} M EDTA. With other samples of ferricyanide and buffer, including some of pH 5.6, similar results were obtained but the inflection point occurred at different EDTA concentrations.

Another set of experiments was done with cysteine (I), ferricyanide (I), and acetate buffer (III), the reagents used in obtaining the data represented in Fig. 2. With no EDTA added, the ferricyanide absorbance remaining after 45 sec was negligible; with 2×10^{-6} M EDTA, a little more than corresponds to the inflection point, the absorbance after 45 sec was 0.261, corresponding to 34% reaction; with 4×10^{-6} M EDTA, the absorbance was 0.375. Now, several aliquot portions of the cysteine solution containing 4×10^{-6} M EDTA were taken, and to each was added a metal salt in the amount needed to give 2×10^{-6} M concentration; then ferricyanide was added, and the absorbance after 45 sec was measured. A representative set of results is given in Table AI-1.

Figure 3 represents data obtained with 4×10^{-3} M 3-mercaptopropionic acid and 4×10^{-4} M ferricyanide in acetate buffer of pH 4.0,

TABLE AI-1

EFFECT OF METAL SALTS ON REACTION

Added Substance	Absorbance
$CuSO_4$	0.005
$FeSO_4$	0.005
$Fe_2(SO_4)_3$	0.014
$CoCl_2$	0.005
$SnCl_2$	0.005
$MnSO_4$	0.313
$Ni(NO_3)_2$	0.220
$CrCl_3$	0.245

plotted so as to give a straight line if the rate of consumption of ferricyanide were of second order (16). Figure 4 shows the data obtained with 4×10^{-3} M 1-octanethiol in acetone-water-carbonate buffer, plotted so as to give a straight line if the rate of consumption of ferricyanide were of first order; a second order rate constant can be calculated from the experimental first order constant by dividing into it the mercaptan concentration.

Figure 5 represents the dependence of half-reaction times on EDTA concentration for cysteine (I) and for 3-mercaptopropionic acid. Figure 6 represents the dependence of calculated second order rate constants on EDTA concentration for octanethiol. It must be stressed that different samples of chemicals might be expected to give quantitatively different results though the shape of the curves might be similar.

DISCUSSION

The variation in rates of reaction observed with different samples of ferricyanide, cysteine, and buffer salts, as well as the inhibitory effect of EDTA on the reaction, can be reasonably explained by the hypothesis that the reaction is catalyzed by traces of metal ions which are present as impurities.

If EDTA combines with the catalyst to form a slightly dissociated complex, the concentration of free catalyst should change very rapidly near the equivalence point and cause a corresponding shapr inflection in the rate of reaction. Just such a situation is represented in Fig. 2. The inflection point, at about 1.9×10^{-6} M, is presumably in stoichiometric correspondence to the concentration of catalyst present in this case. Since the concentration of solid reagents, mostly buffer

salts, was more than 0.2 M, this amount of impurity corresponds to only 0.001 mole %, not an unreasonable value. The fact that similar behavior was found with other smaples of chemicals but that the inflection occurred at different EDTA concentrations is consistent with the proposed interpretation.

What is the catalyst present in these samples? It is natural to suspect the ions of iron, because they are known to act as catalysts in other oxidation processes, for instance in the reaction of cysteine with oxygen, and also because they are of sufficiently widespread occurrence that they may well be present in the reagents used to the extent indicated (98). When sufficient EDTA was added to inhibit the reaction almost completely, adding ferrous or ferric ions restored the reaction, which is consistent with the suspicion expressed above. However, the demonstration is not unambiguous, because it could be that the iron added displaced the original catalyst from its EDTA complex. Also, other ions, namely copper(II), tin(II), and cobalt(II) had an effect equal to iron (insofar as this could be tested by the experiment). It is not likely that the latter two ions would have been present in the original reagents, but copper(II) might have been. It is clear that the question cannot be settled at this time, and that the results of the experiments show only that iron and/or copper could be the catalyst(s) involved.

Presumably, the metal ion mediates the transfer of electrons from mercaptan to ferricyanide. For the reaction of 3-mercaptopropionic acid with ferricyanide, Bohning and Weiss proposed a mechanism which involves three steps, namely oxidation to ($RS\cdot$), then to (RS^+), and reaction of (RS^+) with RSH to give RSSR (16). This mechanism is based

in large part on evidence not considered in the present work, and therefore it will not be discussed critically at this time. So far as the metal-ion catalysis is concerned, it can be introduced into the mechanism by postulating that ferricyanide reacts not with the mercaptide ion but with a metal-mercaptan complex, e.g., for a divalent ion.

$$RSM^{+} + [Fe(CN)_6]^{3-} \rightarrow RS\cdot + [Fe(CN)_6]^{4-} + M^{2+}$$

In the interpretation of the kinetics of oxidation of 1-octanethiol, Kolthoff et al., (62) found that cyanide inhibits the reaction and postulated that the effect comes about because cyanide repressed the equilibrium.

$$[Fe(CN)_6]^{3-} + RS^{-} \rightleftharpoons [Fe(CN)_5RS]^{3-} + CN^{-}$$

However, there is a striking similarity between the inhibition by cyanide (see Fig. 6, ref. 62) and the results obtained in this work with EDTA (Fig. 6). Since cyanide is a good complexing agent for iron and copper ions, as is EDTA, it seems logical to suggest that the inhibition is due in both cases to complexation of catalytic impurities present in the reaction mixtures.

ACKNOWLEDGMENTS

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the summer 1962 by Mr. M. E. Bell, a participant of the Research Participation Program sponsored by the National Science Foundation, and by Dr. J. Leslie.

AI-1

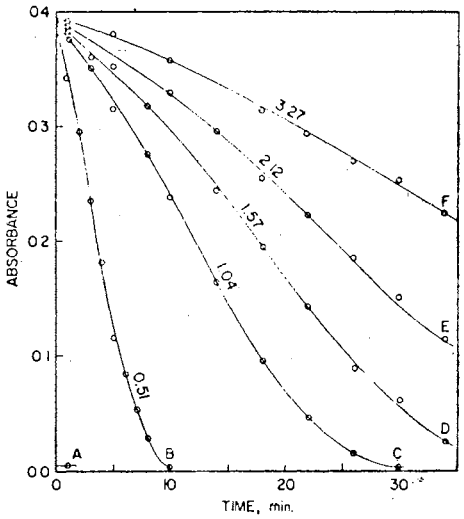

FIG. 1. Rate of reaction of ferricyanide (I), initially $4 \times 10^{-4} M$, with cysteine (I), initially $3.8 \times 10^{-3} M$, in acetate buffer (II) of pH 4.0. Curve A: no EDTA; curves B-F: EDTA concentration 0.51 to $3.27 \times 10^{-5} M$.

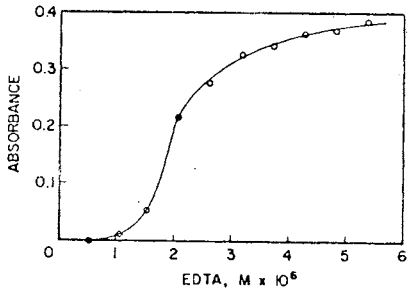

FIG. 2. Absorbance at 410 mμ 45 sec after mixing ferricyanide (I), initially $4 \times 10^{-4} M$, with cysteine (I), initially $3.8 \times 10^{-3} M$, in acetate buffer (III), pH 4.0, in the presence of varying concentration of EDTA.

AI-2

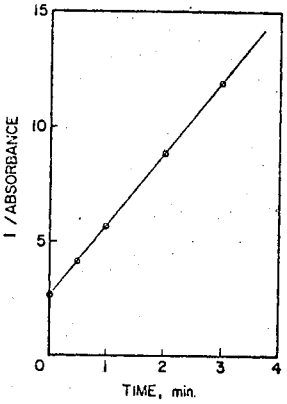

FIG. 3. Rate of reaction of ferricyanide (II), initially $4 \times 10^{-4} M$, with 3-mercaptopropionic acid, initially $4.0 \times 10^{-3} M$, in acetate buffer, pH 4.0.

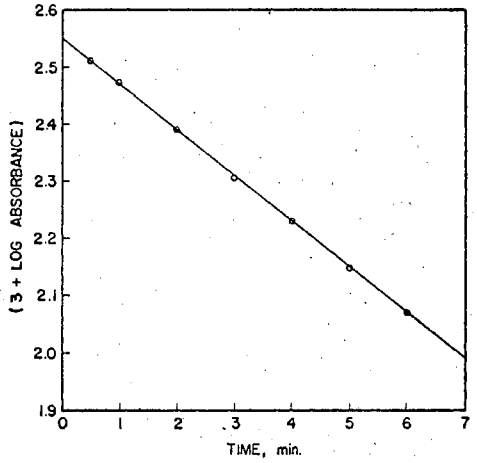

FIG. 4. Rate of reaction of ferricyanide (II) initially $4 \times 10^{-4} M$, with 1-octanethiol, initially $4.0 \times 10^{-3} M$, in acetone-water-carbonate buffer.

AI-3

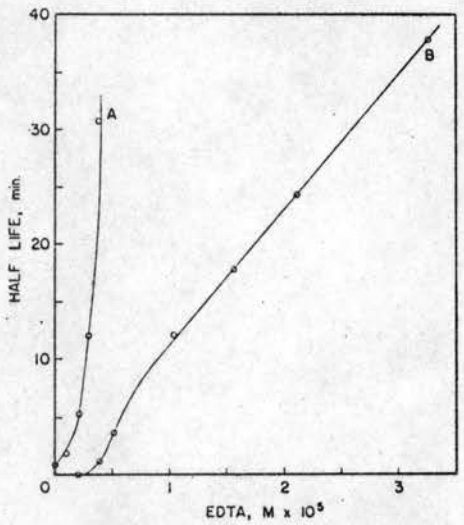

FIG. 5. Dependence of half-reaction times on EDTA concentration. Curve A: 3-mercaptopropionic acid; curve B: cysteine (I), ferricyanide (II), acetate buffer (I).

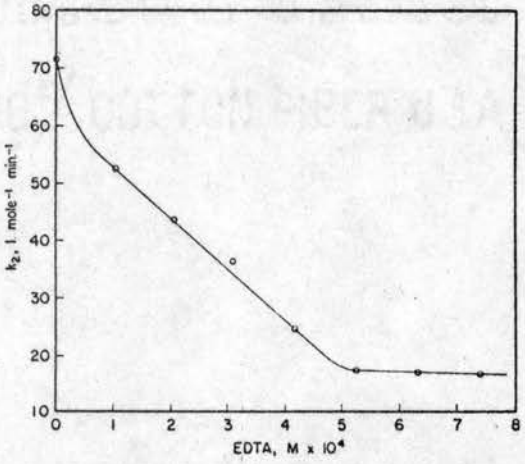

FIG. 6. Dependence of second order rate constant on EDTA concentration for reaction of ferricyanide (II), initially $4 \times 10^{-4} M$, with 1-octanethiol, initially $3.8 \times 10^{3} M$, in acetone-water-carbonate buffer.

APPENDIX B

THE OXIDATION OF CYSTEINE AND CYSTINE TO CYSTEIC ACID WITH IODATE

ABSTRACT

The reaction stoichiometry, approximate rates and, in some cases, the nature of the products have been determined for the reaction of iodate with cyst(e)ine at various absolute and relative concentrations in 0.1-1 M hydrochloric acid and at pH 4 and 7. In 1 M hydrochloric acid and with excess iodate cysteic acid is formed rapidly and quantitatively. In most other conditions, mixtures of products are obtained. The results indicate that cystine is not an intermediate in the oxidation of cysteine to higher oxidation products; on the contrary, cystine is formed by a competing reaction pathway and, once formed, may block further oxidation.

INTRODUCTION

The oxidative scission of the disulfide bond of cystine to cysteic acid is a procedure widely used in the investigation of protein structure (20). The reagent commonly used for this conversion is performic acid (103). The characteristics of this reagent are not ideal, and the present study was undertaken in the hope that iodate could be used to effect the aforesaid reaction. Potassium iodate is readily available in a state of high purity and quite stable; in these respects at least it would be advantageous to use.

The extant literature contains surprisingly little information

about the reaction. An abstract of a paper presented by Lavine (65) mentions that iodate in 1 N hydrochloric or sulfuric acid oxidizes cysteine and cystine to cysteic acid, but no paper on the subject was published. Hird and Yates (54) measured the reaction of iodate with cysteine at pH 7 and state that "10% higher products [higher than cystine]" were formed. Williams and Woods (134) report that cystine reacts with iodate in 40% sulfuric acid at 185° but the product was not determined.

EXPERIMENTAL

Materials. Two samples of L-cysteine hydrochloride monohydrate (Calbiochem, A-grade, lot 36293, and B-grade, lot 103993) and of L-cystine (Schwarz Bioresearch, lot CSL570, and Matheson, Coleman and Bell, lot 3041308) were used, with the same results. L-Cysteinesulfinic acid was from Schwarz, lot 36B890, cysteic acid from Calbiochem, CFP, lot 400252. All other chemicals were of A.C.S.-reagent grade. The water was obtained from condensation of steam and passed through a column of Rexyn I-300 (Fisher). The pH-4 was 0.16 M in acetic acid and 0.04 M in sodium acetate; the pH-7 buffer 0.0266 M in NaH_2PO_4 and 0.0400 M in Na_2HPO_4.

Determination of Stoichiometry. A weighed sample of cysteine or of cystine was dissolved in 100 ml of acid or buffer. A 25-ml sample of this solution was mixed with 5 ml of iodate solution in the same medium. At timed intervals after mixing, 3-ml aliquots of the reaction mixture were withdrawn and added to a quenching solution consisting of 3 ml of 1 M HCl + 5 ml of 1 M KI + 1 ml of 1% starch indicator + 5 ml of water. The sample was then titrated with thiosulfate; this solution

was standardized against weighed portions of potassium iodate, which was dissolved in the quenching solution. The reactions were conducted at room temperature, 20-4°.

Paper Chromatography. The sample was applied to a 2 x 13-cm strip of Whatman No. 1 filter paper. "Liquefied Phenol" (88%) was allowed to ascend the paper strip to about 10 cm above the point of sample application. The strip was dried, then sprayed with 0.4% ninhydrin + 1.5% 2,4,6-trimethylpyridine in 95% ethanol. The R_f values (measured to the leading edge of the spots) were: cystine 0.33; cysteinesulfinic acid 0.22; and cysteic acid 0.12.

STOICHIOMETRIC CONSIDERATIONS

The equations that describe the reaction of iodate with iodide in strong acid and the backtitration with thiosulfate are:

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

$$I_2 + 2S_2O_3^{=} \longrightarrow 2I^- + S_4O_6^{=}$$

In evaluating the reaction of iodate with cyst(e)ine, it will be convenient to express the results in terms of the number of electrons Q transferred from each molecule of the reagent to the oxidant. This is given by the expression:

$$Q = (V_b - V_s)M/n$$

where V_s is the volume in milliliters of thiosulfate of molarity M consumed by a sample containing n millimoles of cyst(e)ine and V_b the volume consumed by the iodate blank to which no cyst(e)ine had been added. The value of Q does not depend on the nature of the

iodine-containing reduction product, inasmuch as the final product is iodide in every case. For example, consider the oxidation of cysteine (RSH) to cysteic acid (RSO_3H); this conversion involves the removal of six electrons, as can be seen by writing the partial equation:

$$RSH + 3H_2O \longrightarrow RSO_3H + 6H^+ + 6e \qquad \text{(AII-1)}$$

Now consider three possible stoichiometric equations for this reaction:

$$RSH + IO_3^- \longrightarrow RSO_3H + I^-; \qquad \text{(AII-2)}$$

$$5RSH + 6IO_3^- + 6H^+ \longrightarrow 5RSO_3H + 3H_2O + 3I_2; \qquad \text{(AII-3)}$$

$$2RSH + 3IO_3^- \longrightarrow 2RSO_3H + 3IO^- . \qquad \text{(AII-4)}$$

In case (AII-2), the blank would consume 6 moles of thiosulfate and the reaction mixture none, since the product is iodide; hence $\underline{Q} = 6$. In case (AII-3), the blank would consume 36 moles of thiosulfate and the reaction product 6, for 5 moles of cysteine; hence, $\underline{Q} = (36 - 6)/5 = 6$. In case (AII-4), the blank would consume 18 moles of thiosulfate; on quenching the reaction mixture, $3IO^- + 3I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$, and the liberated iodine would consume 6 moles of thiosulfate; hence, again, $\underline{Q} = (18 - 6)/2 = 6$.

It should be understood that equations (2-4) are at this point hypothetical. It will be seen below that the concentrations, pH, etc. determine what actually happens; $\underline{Q}$ is <u>in fact</u> 6 only in certain conditions, and only in those conditions could cysteic acid have been formed quantitatively.

Table AII-1 gives the values of $\underline{Q}$ theoretically required to form various possible products from cyst(e)ine; they are derived by writing balanced partial equations similar to (AII-1). With reference to the

TABLE AII-1

ELECTRONS REMOVED (= Q VALUES) IN OXIDATION TO VARIOUS PRODUCTS

Product	From Cysteine (RSH)	From Cystine (RSSR)
RSSR	1	-
RSOSOR	3	4
RSO_2SO_2R	5	8
RSO_2H	4	6
RSO_3H	6	10

table, the argument can now be applied in reverse. If, for example, cysteine is oxidized and the experimental value of Q is 1, the product must be cystine. If the value of Q is 4, the product might be cysteine-sulfinic acid; but it will be obvious that the same stoichiometric relationship would exist if, instead, a mixture of cystine and cysteic acid were formed in 2:3 molar ratio.

RESULTS

The following concentrations were employed in all the media tested: 10^{-3} M cysteine or cystine and 2.4×10^{-3} M iodate; this proportion is more than that required for oxidation to cysteic acid if the iodate is reduced to iodine. The results reported below refer to these concentrations, except when other specifications are stated. Aliquot portions of the reaction mixtures were analyzed at intervals; no systematic study of the kinetics was made, however, and only representative data are presented. In selected cases, the product was subjected to paper chromatography.

Figure AII-1, curves A and B, represent data obtained with cystine in 1 M and 0.1 M hydrochloric acid, respectively. The average Q obtained in several experiments was 10.0 ± 0.1 in 1 M acid, 9.9 ± 0.1 in 0.1 M acid; the same stoichiometry was found as the cystine concentration was varied from 10^{-2} to 10^{-4} M, iodate being taken in 2.4-fold molar ratio in each case. The rates were too fast for accurate measurement by the method employed, that gave significant results only for the latter stages of the reaction. It is qualitatively clear, however, that the rate 0.1 M acid was considerably slower; if 10^{-3} M iodide was added to the cystine solution in 0.1 M acid prior to the addition of

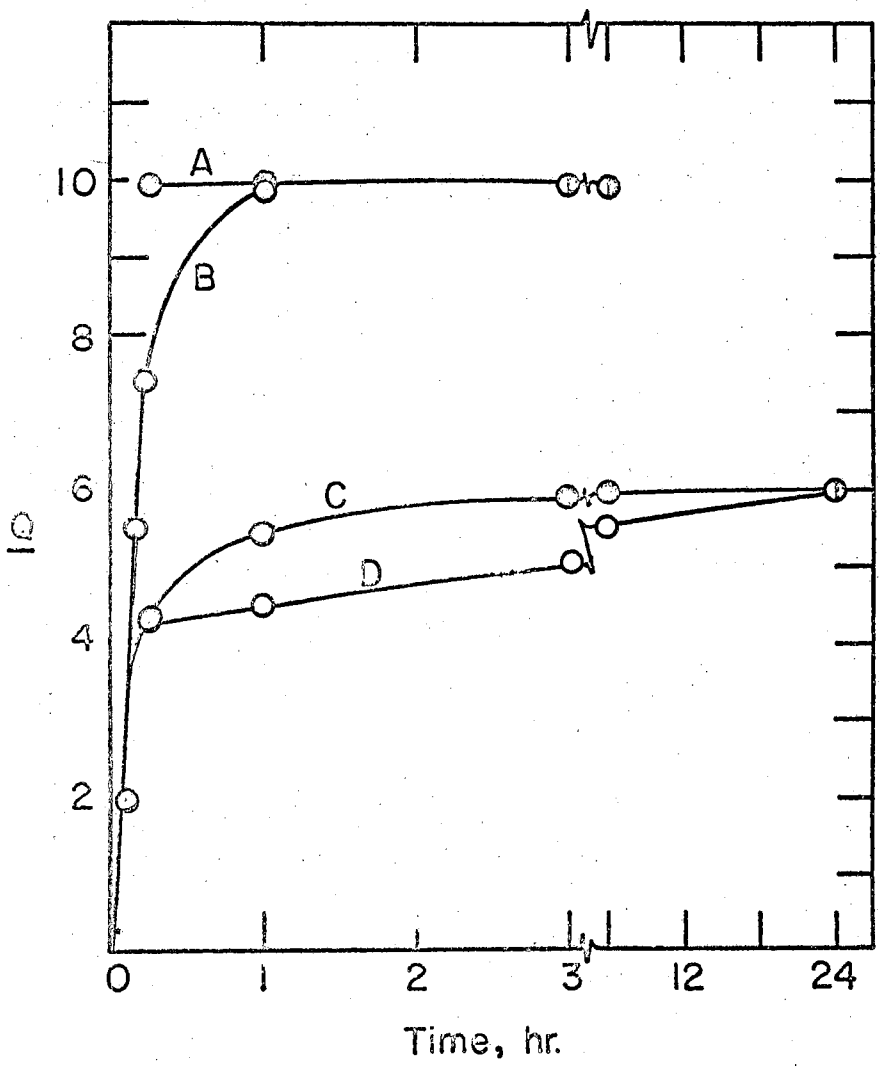

Fig. AII-1. Reactions of 2.4 x 10^{-3}M Iodate with 1 x 10^{-3}M Cysteine and Cystine in Acid Solution. A-Cystine in 1M HCl; B-Cystine in 0.1M HCl; C-Cysteine in 1M HCl; D-Cysteine in 0.1M HCl.

iodate, the rate became as fast as in 1 M acid.

The product of the reaction was subjected to paper chromatography. In one case, the initial concentration of cystine was 10^{-2} and iodate 2.4×10^{-2} M; only one ninhydrin-positive spot was obtained, with R_f identical to that of an authentic sample of cysteic acid. In another experiment, 10^{-2} M, cystine was reacted with iodate in 1:1 molar ratio; two spots were then found, corresponding to cysteic acid and unreacted cystine, and no spot was obtained with the R_f value of cysteinesulfinic acid (because the cystine spot "tailed" small amounts of the former might not be detected; however, the formation of cysteinesulfinic acid as an important product could also be excluded on stoichiometric grounds, since the amount of cysteic acid formed, as estimated from the intensity of the spot, accounted approximately for the iodate consumed.)

Representative data obtained with cysteine in 1 M and 0.1 M hydrochloric acid are shown in Figure AII-1, curves C and D. The average value of Q was 6.0 ± 0.1 in 1 M acid and 5.9 ± 0.1 in 0.1 M acid. For the reaction in 0.1 M acid, two stages can be discerned, a fast initial consumption of iodide, followed by a slower reaction. Chromatography of the product formed from 10^{-2} M cysteine and iodate in 1:2.4 molar ratio showed the presence only of cysteic acid. With cysteine and iodate in 3:2 molar ratio, cysteinesulfinic and cysteic acid were the products; with 6:1 molar ratio, cystine and cysteinesulfinic acid were the principal products, but a small amount of cysteic acid also was found.

In acetate buffer of pH 4, cystine reacted extremely slowly: Q was <0.5 in 24 hr. The reaction was catalyzed by iodide, and the presence of 10^{-3} M added iodide the consumption of iodate was quite

appreciable, albeit still comparatively slow; a Q value of 1.2 was attained in 6 hr., 4.6 in 24 hr.

The course of the reaction of cysteine in pH 4 buffer again consisted of two stages, proceeding at very different rates. The Q value attained in the initial, fast stage was very dependent on the cysteine-iodate ratio; curves A and B in Figure AII-2 illustrate two cases, in which Q was 3.3 and 1.8, respectively. The value attained in the former case approached that required for formation of cysteinesulfinic acid, but paper chromatography of the product showed that this was not in fact produced (except possibly in small amounts, which would not be detected): cystine and cysteic acid were the two products found.

In phosphate buffer of pH 7, cystine did not react with iodate. Cysteine reacted rapidly, typically as is represented by curve C in Figure AII-2; in this case, Q = 1.63. At other absolute and relative concentrations, 2.5 to 5 x 10^{-3} M cysteine and iodate in 1:2.5-5 molar ratio, Q varied between 1.5 and 2; in every case, the value quoted is the limiting value, which was reached quickly and then remained quite constant with time.

As has been seen, cysteinesulfinic acid is a product in some cases and a probable intermediate in others. It is therefore of interest to know how it reacts with iodate. In 1 M hydrochloric acid, the value of Q = 2 was attained in less than 5 min.; this of course is the value required for the conversion to cysteic acid. In acetate buffer of pH 4 with 10^{-3} M added iodide, some iodate was consumed very quickly but stoichiometric completion was attained only after about 6 hr; in the absence of added iodide, Q values were 1. Apparently, the reaction attains fairly quickly a state of quasi-equilibrium, after which the

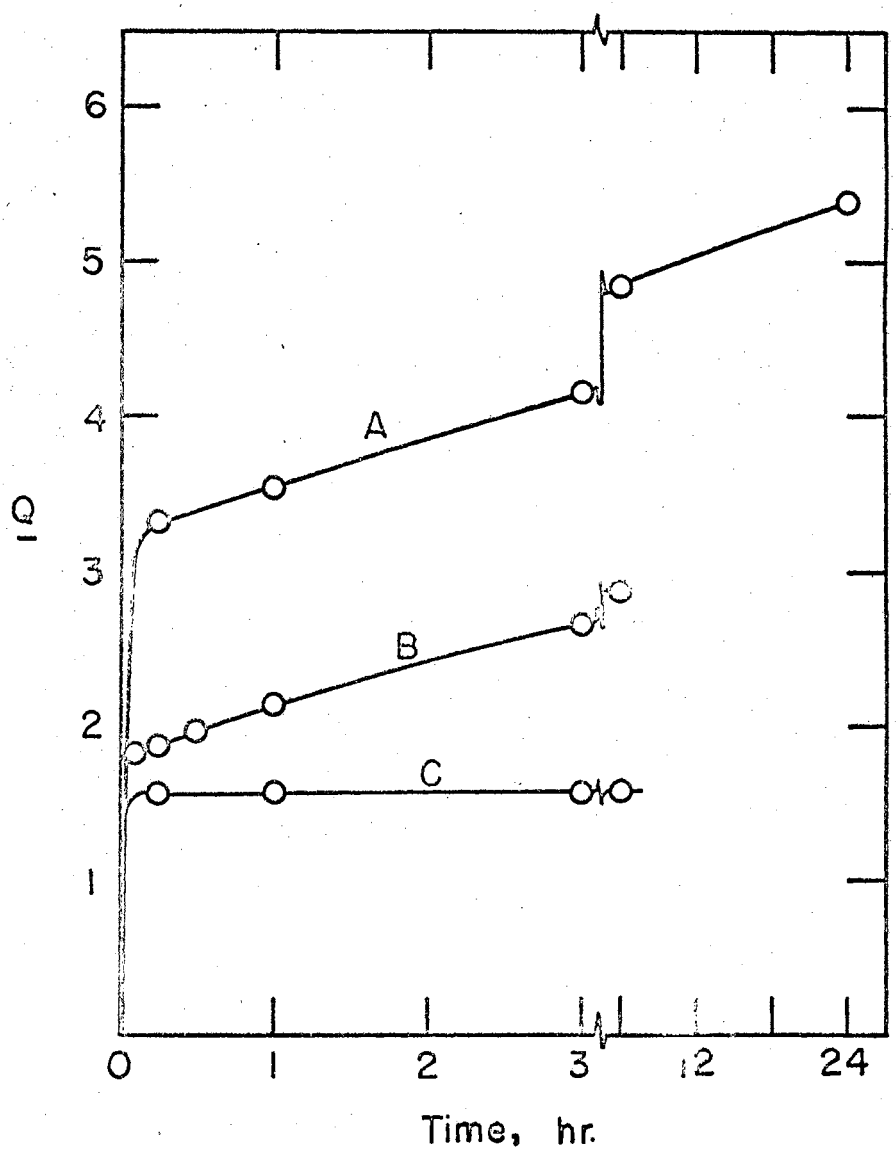

Fig. AII-2. Reactions of 2.4 x 10^{-3}M Iodate with Cysteine at pH 4 and 7. A - 1 x 10^{-3}M Cysteine in pH 4 buffer; B - 5 x 10^{-3}M Cysteine in pH 4 buffer; C - 1 x 10^{-3}M Cysteine in pH 7 buffer.

reaction proceeds slowly.

In all the experiments, when iodate was mixed with cyst(e)ine, the brown color that is characteristic of aqueous iodine solutions developed and in general intensified as the reaction proceeded. In many cases, however, the intensity of color and its development with time did not correspond with the amount of iodate reduction that was found analytically after quenching. This indicates that species of intermediate oxidation number, such as HIO and possibly HIO_2 and, at higher pH, the corresponding salts, were formed as products or as fairly stable intermediates.

DISCUSSION

There is good reason to anticipate that the reactions of cysteine and cystine might be quite complex, since a number of products, such as those listed in Table AII-1, might be formed. The reduction of iodate, for its part, involves the transfer of several electrons, five in the conversion to I^o, and also must involve a number of intermediates. It may be expected that determination of the kinetics of the reactions and elucidation of their mechanisms will be a very interesting problem, but possibly also a quite difficult one. The principal concern of this paper is the simpler question: what products are formed from cysteine and cystine? As explained in the section on Stoichiometric Considerations, the use of Q values allows one to focus on this question, without regard to the nature of reduction products.

Reference was made in the Introduction to the potential usefulness of a procedure for converting cystine to cysteic acid. The results of the present work show that in 0.1-1 M hydrochloric acid the reaction

can be realized conveniently and quantitatively. The catalytic effect of added iodide, clearly demonstrable in 0.1 M acid, leaves no doubt that species of intermediate valence play an important role in the kinetics of the reaction. The reaction proceeds most rapidly in 1 M acid, and not at all at pH 7. The results do not inform on the point at which cleavage of the S-S bond occurs; there may be a series of intermediates up to the disulfone, which then splits, or cleavage may occur at some earlier stage. Whatever the intermediates may be, cysteic acid is the only demonstrable product in 1 M acid, even with less than equivalent iodate; this indicates that the specific rate of formation of the intermediates is less than that for their further oxidation, so that they do not accumulate.

The conversion of cysteine to cystine is a two-electron oxidation, and it is not difficult to visualize how this might take place by interaction with iodate (or any oxyiodide ion):

$$RSH + IO_3^- \longrightarrow RSOH + IO_2^- \qquad \text{(AII-5)}$$

RSOH could then react with cysteine to give cystine:

$$RSOH + RSH \longrightarrow RSSR + H_2O \qquad \text{(AII-6)}$$

Cystine could also be formed by combination of two thyil radicals which of course may be derived from RS^- by loss of a single electron. Two successive electron removals produces RS^+, which is at the same oxidation level as RSOH; hence it is possible to arrive at reaction (5) by the one-electron route, although reaction (5) would seem to be the more likely path.

The above considerations are entirely speculative and may therefore seem superfluous, however, postulation of an intermediate such as

RSOH makes it easy to rationalize what takes place at pH 4 and 7. In order to produce RSO_2H and RSO_3H, there must of course be further interaction with the oxidant, and the final proportions of RSSR and of higher products will therefore depend on the outcome of the competition between the oxidant and RSH. At pH 7, cystine formation predominates, and increasing the iodate concentration raises Q by only a limited, but appreciable, amount. When the cysteine is exhausted, the reaction comes to an abrupt stop, since cystine is not oxidized in the conditions. This explains the course of the reaction, which seemed strange when it was first observed.

Comparison of the results at pH 4 in dictate that the pathway leading to higher oxidation products is relatively favored, which seems quite reasonable; at comparable concentrations, Q is nearly 4 rather than nearly 2. However, Q now responds more drastically to reduction of the iodate ratio; at low ratios, cysteinesulfinic acid is an important product. This of course is consistent with the view that cysteinesulfinic acid is an intermediate in the conversion of cysteine to cysteic acid.

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